Human health implications of exposure to xenoestrogens from food

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By

Barbara Mary Thomson

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Dedicated to the memory of my mother

Dulcie Oulaghan

1909-1982

“ Science is built of facts the way a house is built of bricks;
but an accumulation of facts is no more a science than a pile of bricks is a house”
-Henri Poincaré, La Science et l’hypothèse

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Abstract

This thesis aims to assess the human health impact of exposure to estrogenic compounds from the diet. A multi-disciplinary approach is taken to address various aspects of this issue. An introduction to xenoestrogens, including international research priorities, wildlife and human health effects, mechanisms of action, structure activity relationships and additivity of estrogenic effects is provided as background information.

An assessment of exposure to a range of naturally occurring and synthetic estrogenic compounds found in food is derived in Chapter 2. The assessment combines new and existing data on food concentration, food consumption and serum levels for each xenoestrogen. Exposure is combined with relative estrogenic potency data from published bioassay data to estimate risk relative to normal circulating levels of estradiol. Assuming additivity of xenoestrogens, for an average New Zealand male and for post-menopausal women, xenoestrogens in the diet contribute an additional 12-90% of estrogenicity above normal circulating levels. For a pre-menopausal female, the contribution from the diet represents in the order of an additional 2%. The level of exposure determined in this thesis would seem to be of pharmacological relevance, especially for men with low levels of estrogen and for post-menopausal women.

Bisphenol A (BPA) is an important monomer used in the manufacture of epoxy resins for internal food can linings. A survey of the BPA content of a range of 80 canned foods available to the New Zealand consumer was undertaken and the results used in the exposure and risk assessments. BPA was detected in all foods analysed except soft drinks, at concentrations ranging from <10-29 μg/kg, except for individual samples of tuna, corned beef and coconut cream that were 109, 98 and 191 μg/kg respectively. None, of over 4000 individual exposure scenarios, exceeded the temporary Tolerable Daily Intake (TDI) of 10 μg/kg body weight per day set by the Scientific Committee on Food in 2002.

Intestinal microflora influence the bioavailability of the naturally occurring xenoestrogens genistein and daidzein that contribute significantly to total estrogenicity from the diet. The

degradation of genistein and daidzein by the faecal microfloral of 5 human subjects was variable and unpredictable between individuals and within an individual. These findings have important implications for the promotion and prescription of soy foods and supplements for disease prevention and health benefits.

The "yeast assay" is one of a number of methods available to measure estrogenicity. This assay was established and validated. *In utero* exposure to estrogenic compounds at critical periods of sexual differentiation and endocrine development may imprint for health effects observed later in life. Placental transfer of estrogenicity, from 17β-estradiol was studied using the human placental perfusion model and the yeast assay. The placenta provides a protective barrier to the transfer of estrogenicity. Experiments with genistein showed that 5-15% placental transfer occurred, suggesting that *in utero* exposure might be in the order of 10% of maternal exposure.

The thesis concludes with consideration of a genomic approach to substantiate, or refute, the mechanistic link between exposure to xenoestrogens and claimed human health effect. Such an approach offers exciting opportunity to clarify the mode of action of the synthetic versus the naturally occurring xenoestrogens, to confirm or dispute additivity of effect that is an important premise of the exposure assessment, to identify key genes involved in the many possible health effects and thence risk to the individual from dietary exposure to xenoestrogens.

Scientific communications from this thesis.

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Thomson BM, Cressey PT, Shaw IC (2003) Dietary Exposure to Xenoestrogens in New Zealand, Journal of Environmental Monitoring; 5: 229-235*s*

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BM Thomson*, PJ Cressey, IC Shaw. *Should real men eat soy?.* Oral presentation at the annual conference of the New Zealand Institute of Food Science and Technology, Dunedin, August 2001.

BM Thomson*, PJ Cressey, IC Shaw. *The impact of dietary xenoestrogens for men.* Oral presentation at the 8th ICEM, Japan, October 21-26, 2001. Mutation Research; 483 (2001): S1-S192.

BM Thomson*, PJ Cressey, NJ Turner and IC Shaw. *Human dietary exposure to xenoestrogens in New Zealand,* Oral presentation at the Society of Environmental Toxicology and Chemistry/Australasian Society for Ecotoxicology International conference, Christchurch, New Zealand 28 September 2003.

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E Podivinsky and **B Thomson,** Environmental estrogen exposure and prostate cancer . Poster presentation at Medical Sciences Congress, Queenstown, 30 Nov-3 Dec, 2004.

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E. Podivinsky* and **B.M Thomson***, *Boys, Barbeques, Baked Beans and Babies.* Seminar to the School of Biological Sciences, University of Canterbury, October 6, 2004.

BM Thomson* and E Podivinsky**,** *Boys, Baked beans and Babies*. Joint meeting of the New Zealand Institute of Chemistry and New Zealand Institute of Food Science and Technology. Christchurch, October 20th, 2004.

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Abbreviations and units

AGI	anogenital index (a measurement from the centre of the anus to the base of the penis)
A:T ratio	the ratio of actual (measured) versus theoretical (estimated from intake) serum levels
BHA	butylated hydroxyanisole
BPA	bisphenol A
CREDO	Cluster of Research on Endocrine Disruption
CPRG	chlorophenol red-β-D-galactopyranoside
DDE	dichlorodiphenyldichloroethene
DDT	dichlorodiphenyltrichloroethane, including *o,p'- and p,p'*-isomers
DES	diethylstilbestrol
EC_{50}	the concentration at which half a maximum effect is observed
EDC	endocrine disrupting chemical
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	estrogen receptor
ERE	estrogen responsive element
ERMA	Environmental Risk Management Agency
EU	European Union
GCMS	gas chromatography-mass spectrometry
GnRH	Gonadotropin-releasing hormone
GTT	gut transit time
hCG	human chorionic ganadotrophin
HCH	hexachlorocyclohexane
ILO	International Labour Organization
IPCS	International Programme on Chemical Safety
LOD	limit of detection
LOQ	limit of quantitation
MfE	Ministry for the Environment
NIH	National Institutes of Health
NNS	National Nutrition Survey

NZ	New Zealand
NZTDS	New Zealand Total Diet Survey
OC	organochlorine
ORD	Office of Research and Development
PCB	polychlorinated biphenyl
SAR	structure activity relationship
SHBG	sex hormone-binding globulin
TDE	tetrachlorodiphenylethane
TDI	tolerable daily intake
TEF	toxic equivalent factor
UNEP	United Nations Environment Programme
XEQ	the estrogenicity of a xenoestrogens relative to 17β-estradiol
YES	yeast estrogen screen
WHO	World Health Organisation

l	litre, a measure of volume
mg/day	milligrams per day, a measure of daily dose or intake
mg/kg	milligrams per kilogram, a measure of concentration
nmol/l	nanomoles per litre, a measure of concentration
µg	micrograms, a measure of weight
µg/l	micrograms per litre, a measure of concentration per unit of volume
µg/kg bw/day	micrograms per kilogram body weight per day, a measure of daily dose or intake per unit weight
µmol/l	micromoles per litre, a measure of concentration

Chapter 1

Introduction to xenoestrogens and their actions

1.1 Background and history of xenoestrogens

The endocrine systems of the body regulate metabolic processes including nutritional, behavioural, reproductive, growth, gut cardiovascular and kidney function (WHO, 2002a). The fundamental role of all endocrine systems is to enable a regulated response of target tissue to a signal originating elsewhere and to maintain homeostasis, avoiding big fluctuations in response. This is mediated by feedback signals from target organs to the regulating cells. All the endocrine systems of the body are integrated via cross-talk. For example, reproductive function needs to account for age and nutritional status. The result is that an environmental compound may affect several endocrine systems such as reproductive tissue, bone growth and cardiovascular function. For many endocrine systems, homeostatis programming is established during fetal/neonatal development so that an imbalance at this stage of life, perhaps from an environmental compound, may result in permanent misprogramming (Seckl and Meaney, 2004, Owen *et al.,* 2005).

Hormones are the chemical messengers of the endocrine system. Hormones are released into the bloodstream from numerous glands and tissues (Figure 1.1).

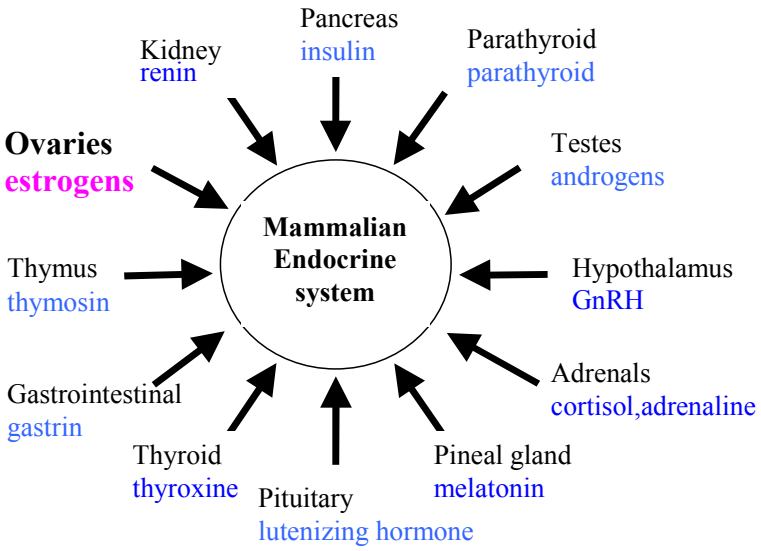


Figure 1.1: Hormone producing glands and tissues of the endocrine system (Norris, 1996) with example hormones (in colour).

The endocrine system is integrated and complex with interactions between different components. For example, production of testosterone from Leydig cells in the testis is stimulated by luteinizing hormone produced in the pituitary gland that in turn is controlled by the peptide gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus (WHO, 2002a). Both the male and female sex hormones (androgens and estrogens) are synthesized from a common precursor, cholesterol, via the intermediate pregnenolone. Interestingly, mammalian estrogen biosynthesis requires prior synthesis of an androgen, or an external source of androgen. In addition the androgen, testosterone, may be converted to estrogen in the brain (Norris, 1996).

A cell may be exposed to both endogenous and exogenous sex hormones, or hormone mimicking compounds, the level of which is influenced by various enzymes such as P_{450} Phase I, α-reductase and aromatase for example. The complexity of contributing sex hormones at a cellular level is illustrated in Figure 1.2. Whilst recognising that no one hormone functions in isolation from other hormones of the endocrine system, this thesis focuses solely on the estrogens.

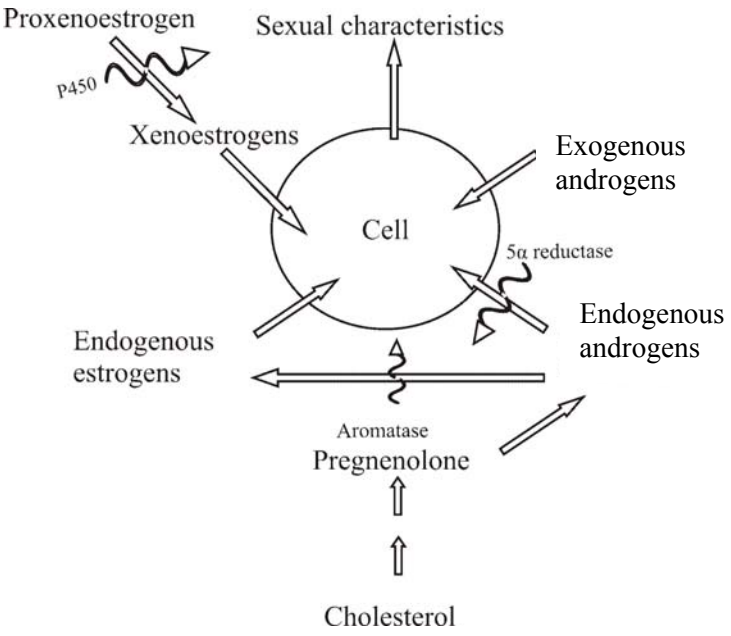


Figure 1.2: The complex interactions of sex hormones in a cellular context.

Estrogens are a family of hormones that promote the development and maintenance of female sex characteristics. The prefix 'xeno' derives from the Greek 'xenos' meaning foreign or strange. Hence 'xenoestrogens' are compounds that are foreign to the body and that mimic the action, at least in part, of estrogens. Xenoestrogens may be synthetic such as the plasticizer bisphenol A (BPA), or naturally occurring compounds, for example genistein that occurs in soy.

The history of xenoestrogens dates back at least to 1938 when Dodds and colleagues synthesized diethylstilbestrol (DES) (Figure 1.3) a chemical with potent estrogenic properties (Dodds *et al.*, 1938). DES has subsequently been used to castrate chickens, increase weight gain in cattle, treat prostate cancer in men, suppress lactation and prevent miscarriage in women (McLauglan *et al*., 2001).

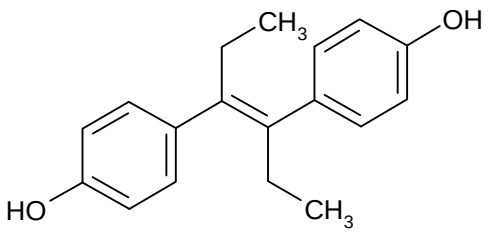


Figure 1.3: Chemical structure of diethylstilbestrol

Plant derived estrogenic compounds (phytoestrogens) were first associated with adverse effects on mammalian development and fertility from observations of animals consuming phytoestrogen-rich plants in the 1940s. Ewes feeding on clover in Western Australia developed abnormal plasma concentrations of endogenous hormones with subsequent loss of fertility (Bennett *et al*., 1946, Moersch *et al*., 1967, Obst and Seamark, 1975). This syndrome was termed "Clover Disease". Subsequent effects on fertility by a known estrogen led to the identification of the naturally occurring xenoestrogens; formononetin, biochanin A and genistein in 3 clover species (Shutt, 1976). Modified farming practices have since prevented further cases of "Clover Disease" (Little, 1996).

The ability of some pesticides to induce estrogen-like responses in the reproductive tissues of rats, chickens and quail has been known since 1968 (Bitman *et al*., 1968). Later, in 1993, Krishnan and colleagues, raised awareness of the potential environmental impact of another synthetic estrogenic chemical used in manufactured products (Krishnan *et al.,* 1993). They observed that water autoclaved in polycarbonate flasks exhibited estrogenic activity. The active compound was isolated and identified as BPA (BPA). This work was significant because it showed that compounds could leach from manufactured products into the environment.

Rachel Carson's book *Silent Spring* (Carson 1962) did much to raise the awareness that chemicals in the environment can exert profound and deleterious effects on wildlife populations and that human health is inextricably linked to the health of the environment. This awareness coupled with the publication by Herbst and colleagues (Herbst *et al.,* 1971) a decade later, that linked maternal ingestion of an estrogenic drug, DES, during early pregnancy with an increased risk of cervical and vaginal cancers in the daughters of exposed mothers, fuelled concern, research and debate over chemicals with hormonal effects -endocrine disrupting chemicals (EDC)s. Xenoestrogens are a major subset of this group of compounds.

Public interest was heightened in the 1990s with:

- the publication in 1992 of a scientific paper by Carlsen *et al.,* (1992) concerning apparent declines in human sperm count.
- publication of "*Our stolen future"* by Theo Colborn in 1996 and
- the broadcast of the 1993 BBC Horizon documentary "*Assault on the male"* .

1.2 International efforts

The growing concern from different groups around the world lead to a major international effort culminating in 2002 with the publication of a "*Global assessment of the state of the science of endocrine disruptors*". This assessment, beginning in 1997, was lead by the International Programme on Chemical Safety (IPCS) that is a collaboration of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organisation (WHO). The steering group was truly international with scientific experts from the USA, United Kingdom, Sweden, The Netherlands, Mexico, Canada, Germany, Japan, France, Italy, and Switzerland. They concluded that analysis of the human data, while generating concerns, does not provide firm evidence of direct causal associations between low–level (as found in the general population) exposure to EDCs and adverse human health effects (WHO, 2002a). The identification of life stages that are most vulnerable to the effects of EDCs and an understanding of how EDC toxicity may affect individual populations were highlighted as research priorities.

A second global project jointly undertaken by SCOPE (Scientific Committee of Problems of the Environment) and IUPAC (International Union of Pure and Applied Chemistry) culminated in a symposium on "Implications of endocrine active substances for humans and wildlife" in Yokohama, Japan in November 2002a. Scientists (408), managers and public policy-makers from 31 countries met together to share papers on (1) human effects, (2) wildlife effects, (3) exposure assessment, and (4) testing for endocrine active substances and effects. These papers, plus discussion from 6 workshops, provide a view of current research efforts and have been published in a special issue of Pure and Applied Chemistry (SCOPE/IUPAC,2003).

In April 2003, the European Union launched a 4-year, $23 million programme for a new Cluster of Research on Endocrine Disruption (CREDO) (Lorenz, 2003). CREDO involves 60 laboratories across Europe. In line with the findings of the Global Assessment (WHO, 2002a), two areas of interest for CREDO focus on: how hormone-mimicking chemicals interact with each other, particularly at low doses, and to what extent these compounds influence gene expression in non-reproductive organs. The latter approach is a step towards substantiating cause and effect.

In 1996, the US Environmental Protection Agency's (EPA) Office of Research and Development (ORD) identified endocrine disruption as one of its top-six research priorities and developed a risk-based research approach to address some of these uncertainties (USEPA, 2003). The EPA Federal Research Project Inventory includes projects in three categories:

- Methods research that identifies human health and ecological hazards and biomarker research
- Model research that develops risk models, supports basic research that may lead to model development
- Measurement research to quantify exposure or effect (USEPA, 2004).

In 2003, the EPA called for proposals to research high-throughput screening systems to enable the prioritization of chemicals for their potential as endocrine disruptors. This call for proposals was driven by the Food Quality Protection Act of 1996 and the Safe Drinking Water Act Amendments of 1996 that both contain provisions related to determining whether pesticides or chemical substances found in or on food or in drinking water sources may have estrogenic or other endocrine effects. Candidate chemicals number in the order of 87,000 (EPA, 2003).

In the US, research into human health effects of EDCs is funded by the National Institutes of Health (NIH) incorporating 27 institutes and centres. Both the National Institute of Environmental Health Sciences (NIEHS) and the US National Cancer Institute are currently funding research into the role of environmental exposures, on birth and developmental defects, sterility, breast and testicular cancers. With more of a food focus, the NIH is funding a 5-year, $8 million project, to study the safety of phytoestrogens in response to a proliferation of dietary supplements and food additives containing phytoestrogen extracts (IFT, 2004).

The New Zealand Ministry of Research Science and Technology commissioned a report "*Environmental oestrogens: sources and possible health effects in New Zealand* (MoRST, 1998). The NZ Ministry of Health and subsequently New Zealand Food Safety Authority have funded several projects of xenoestrogens in food (Lauren and Veitch, 1996, Taylor and Burlingame, 1998, Thomson *et al.*, 2003, Thomson, 2005) including the significant

study of human exposure to xenoestrogens (Chapter 2) and BPA (Chapter 3) within this thesis.

1.3 Health effects of xenoestrogens

1.3.1 Wildlife effects

Concern about the effects of xenoestrogens on human health has arisen from the accumulating body of evidence of endocrine disrupting effects in wildlife. These effects vary from subtle changes in the physiology and sexual behaviour of species to permanently altered sexual differentiation. Most of the data come from Europe and North America and include effects on invertebrates, fish, amphibians, reptiles, birds and mammals. Effects on wildlife, globally, have been most usefully reviewed in both the WHO (2002a) and SCOPE/IUPAC, (2003) documents. Examples of potential effects in wildlife include:

- *Invertebrates*: The masculinization of female marine gastropods exposed to tributyl tin oxide (TBTO) in antifouling paints is the most convincing example of an endocrine disrupting effect on an invertebrate. At the molecular level, TBTO interferes with hormone metabolism, most probably by inhibition of aromatase, thereby increasing levels of androgens (Oehlmann J and Schulte-Oehlmann U, 2003). Thus this effect is likely to be via an androgenic rather than an estrogenic pathway.
- *Fish*: Reproductive abnormalities in freshwater fish living downstream of pulp and paper mill effluents and sewage treatment effluents. A multiplicity of causative agents have been suggested, including estrogenic compounds (Jobling and Tyler, 2003). Most recently, in a national survey of 51 river sites in England over one third of the male population of roach (*Rutilus rutilus*) were feminized. (Taylor *et al*., 2004). Histological analysis of 654 male roach showed that 218 of them were intersex, as defined by the simultaneous presence of both testicular and ovarian tissue within the gonads, demonstrating that sexual disruption in roach is widespread in English rivers. Trudeau and colleagues (2005) have shown that

waterborne BPA can modulate transcription of estrogen responsive genes transfected into tadpoles and fish.

- *Amphibians*: Population declines and deformities in amphibians in both pristine and polluted habitats worldwide. Gardiner *et al.,* (2003) demonstrated that deformities in frogs is via a retinoid signalling pathway. The conclusion of the WHO review (2002a) is that there is insufficient data to implicate EDCs, and therefore xenoestrogens, as causative agents for these effects.
- *Reptiles*: One of the most publicized examples of wildlife effects has been the developmental abnormalites observed in alligators (*Alligator mississippiensis*) in Lake Apopka, Florida, USA. In 1980, a stream that feeds Lake Apopka was contaminated with high concentrations of the organochlorine pesticide, dicofol, its metabolites and other compounds after a chemical spill. A 90% decline in alligator numbers followed (Guillette *et al*., 1994). Further elegant studies at polluted and unpolluted sites, and in the laboratory, have associated a variety of developmental abnormalities in endocrine and reproductive systems including shortened penises, depressed testosterone and elevated estrogen levels with exposure to organochlorine pesticides (Guillette *et al.,* 1999, Guillette and Iguchi, 2003).
- *Birds*: Eggshell thinning and altered gonadal development in birds of prey exposed to DDT, resulting in severe population decline. Currently, the most favoured mechanism to explain egg shell thinning in some bird species involves the inhibition of prostaglandins but this remains conjectural (Giesy *et al.,* 2003).
- *Mammals*: Exposure to OC contaminants (PCBs, DDE) has been shown to adversely impact the reproductive and immune function in Baltic seals, resulting in population declines (Reijnders, 1980, Roos *et al.,*1998). Whether this is due to immune effects resulting in higher disease rates or declining reproduction is unclear. Sperm concentrations and sperm motility were reduced in adult rats that had been exposed to xenoestrogen contaminated fish during lactation (Aravindakshan *et al.,* 2004).

Overall, the current scientific knowledge provides evidence that some effects observed in wildlife can be attributed to chemicals that disrupt the endocrine system. However, in nearly all cases the causal link between exposure and endocrine disruption is implied but

not established – there is no clear cause-effect relationship. The dearth of causal links more likely reflects the difficulty in establishing rather than the absence of such links, one of the factors being the very large number (around 100,000) of man-made chemicals in everyday use (Sumpter, 2003).

Endocrine disruption can be via a number of mechanisms (see Chapter 1.4), including estrogenic pathways. For wildlife, an estrogenic mechanism, which might be caused by exposure to a xenoestrogen, is implicated in the effects seen in fish, reptiles and mammals. At the present level of knowledge it is either not known, or is unlikely, that exposure to xenoestrogens would account for effects observed in invertebrates, amphibians and birds.

1.3.2 Human health

The evidence of adverse outcomes in wildlife combined with studies in laboratory animals substantiates our concerns about human health effects. Since the sex and thyroid hormones are major determinants of development and function of the reproductive, central nervous, and immune systems, much of the experimental research to date has focused on the effects of EDCs on these key hormone systems and target tissues. The main areas of: reproduction, neurobehaviour, immune function, and cancer were globally reviewed by WHO (2002a). In addition, a compilation of publications has been published by SCOPE/IUPAC (2003). The area is extensive and a synopsis of the current state of evidence, drawn from these and subsequent publications, is provided below.

1.3.2.1 Reproductive effects

Estrogen has profound effects on the development and function of reproductive organs. For example it stimulates proliferation of epithelial cells in the reproductive tract and mammary gland of females and in the prostate of males (Kushner *et al.,* 2003). Hence the potential for estrogenic compounds to interfere with the reproductive system. Xenoestrogens have been implicated in declining human sperm count and quality, fertility impairment, an increase in spontaneous abortions, a decline in the proportion of male babies and in abnormalities of the male reproductive tract.

A number of studies show variations in sperm count within and between countries (e.g. Carlsen *et al.,* 1992) but there are limitations in the study designs that may bias the data. Most of the studies are retrospective, using subjects recruited for other purposes (eg infertility diagnosis) and may not truly reflect the population status. More recently, it has been shown that exposure to persistent organochlorine pollutants (e.g. PCBs and *p,p'* – DDE) may have a slight negative impact on human sperm chromatin integrity (Rignell-Hydbom *et al.,* 2005). The issue remains controversial and the causal association weak with exposure to xenoestrogens or other EDCs, (WHO, 2002a, Fisch and Golden, 2003).

Human studies have shown a possible link between maternal exposure to contaminated Great Lakes fish and reduced fertility (Buck *et al.,* 1999) and an increased risk of infertility for women working in the agricultural industry (Fuortes *et al.,* 1997). The inference is that PCBs and pesticides might account for this reduced fertility. In separate studies, analysis of blood or serum samples from 2000 American and 50 Southeast Asian women showed a link between PCBs and DDE levels and altered human menstrual cycles, with the implication that this alteration may influence other end points such as fertility, pregnancy and reproductive cancers (Windham *et al.,* 2005, Cooper *et al.,* 2005). In a case-control study, Takeuchi and colleagues (2004) reported a positive correlation between BPA concentration in serum and ovarian disease. Women with ovarian disease had mean serum concentrations of 1ng/ml compared with 0.7 ng/ml for normal women ($p<0.05$).

An increased rate of spontaneous abortion has been reported for subjects exposed to a range of pesticides (Rupta *et al.,* 1991). Although a causal link has not been established, compounds that affect levels of progesterone (a precursor to testosterone and estrogen) are of most interest as a mechanistic explanation (WHO, 2002a). It is unlikely that environmental doses would be sufficient to cause spontaneous abortions.

Increased frequency of abnormalities of the male reproductive tract development, namely cryptochidism and hypospadias have been associated with exposure to EDCs, including xenoestrogens. A notable finding was the increased incidence of hypospadias in sons of women consuming vegetarian diets during pregnancy (North *et al*., 2000). A suggested explanation is increased exposure to phytoestrogens for the vegetarian mothers although this has not yet been substantiated with blood levels of phytoestrogens for vegetarian

versus non-vegetarian mothers. In a recently published case-control study utilizing historical samples (1959-1967) taken at a time when DDT was used, maternal serum levels showed no association of DDT or DDE and hypospadias or cryptorchidism (Bhatia *et al*., 2005*).* Neither of these independent studies substantiates a causal link between xenoestrogen exposure and hypospadias or cryptorchidism.

A number of male reproductive disorders (cryptorchidism, hypospadias, testis cancer and low sperm counts) have been linked as interconnected disorders, collectively known as testicular dysgenesis syndrome, with a common origin in fetal life (Sharpe and Skakkebaek, 1993, 2003). In 1993, these authors hypothesized that decreasing sperm counts and increasing incidence of testis cancer, cryptorchidism and hypospadias might be related to increased exposure to estrogenic compounds in utero (Sharpe and Skakkebaek, 1993). Ten years on, results from animal studies suggest that the low estrogenic activity of environmental estrogens would be insufficient to lead to the observed effects and that attention should be redirected to chemicals that alter endogenous androgen production/ action or on factors that can elevate endogenous estrogens in the fetus (Sharpe and Skakkebaek, 2003).

The onset of puberty is regulated by gonadotrophins that stimulate an increase in estrogen levels in both boys and girls, resulting in the pubertal growth spurt (MacGillvray, 2004). The age of puberty has decreased over the last century, presumably due to improved nutrition but perhaps also due to exposure to EDCs (Teilmann *et al.,* 2002). Environmental chemicals including phthalate plasticizers, p,p'-DDE and polybrominated biphenyls, have been suggested as potential causative agents for precocious puberty (Colon *et al.,* 2000, Krstevska-Konstantinova *et al.,* 2001, Blanck *et al.,*2000) although there is not yet any clear cause-effect relationships in humans. Giampietro and colleagues (2004) found no evidence of precocious puberty in 3 year children who had been fed soy for more than 6 months.

1.3.2.2 Neurobehaviour

The brain contains steroid receptors that make it a potential target for xenoestrogens. Data from human and experimental animal studies indicate that exposure (particularly *in utero*) to a number of environmental chemicals including DES, organochlorine pesticides,

some fungicides (methoxychlor, fenarimol), polychlorinated dibenzyodioxins, PCBs, dibenzofurans can have adverse effects on neurological development (such as growth retardation, impairment of intelligence and emotional instability), neuroendocrine function and behaviour (WHO, 2002a, Shirai and Asamoto, 2003, O'Connor and Chapin, 2003). But the mechanisms of these effects remain to be elucidated. The development of neural sex differences is initiated by estradiol, which activates two processes in male neonates; masculinization, the development of male-type behaviours, and defeminization, the loss of the ability to display female-type behaviours. Most recently, Kudwa and colleagues (2005) have demonstrated that one estrogen receptor sub-type, namely ERβ, is involved in defeminization of neonatal mice and they hypothesize that ERβ plays an essential role in sexual differentiation of the brain and behaviour. Assuming an extrapolation to humans, it follows that environmental chemicals that bind to ERβ, could potentially have a modulating effect on defeminization .

1.3.2.3 Immune function

The immune system is a target for many compounds, including drugs and environmental chemicals. Of a large number of compounds with immunotoxic properties, only a few have been shown to cause immunotoxicity that is mediated through an endocrine-disrupting mechanism. These include the potent estrogenic DES, which has been shown to cause a weak immunological change following *in utero* exposure and the AhR binding PCBs, PCDFs and PCDDs (WHO, 2002a). DES but not the estrogenic pesticide methoxyclor (at doses of 0.5 and 50 mg/kg/day respectively), reduced the level of circulating lymphocytes in pubertal rhesus monkeys (Golub *et al*., 2004). Currently, there is little evidence of xenoestrogens impacting on human immune function.

1.3.2.4 Cancer

Increasing trends of cancer in hormonally sensitive tissues such as breast, endometrial, testicular, prostate and thyroid have been attributed, in part, to widespread exposure of the general population to xenoestrogens.

Breast

The unknown etiology of the majority of breast cancer cases, along with large geographical differences in incidence rates, has heightened concerns to the potential role

of environmental exposures, including xenoestrogens, in breast cancer risk. One group of dietary compounds that have received much attention is the phytoestrogens. *In vitro* studies with phytoestrogens have shown both proliferative and antiproliferative effects depending on the tumour cell type, dose, timing of exposure and the phytoestrogen given (Aldercreutz and Mazur, 1997). Phytoestrogens can act via multiple mechanisms of action, both ER mediated and non-receptor mediated and may be estrogenic and antiestrogenic. The indirect evidence that high consumption of soy products, a rich source of phytoestrogens, is associated with a lower risk of breast cancer, is inconsistent. A meta-analysis (Trock *et al*., 2000) indicated that high soy intake might reduce the risk of developing premenopausal breast cancer but has no effect on post menopausal breast cancer risk. Of nearly 30 studies critiqued by the WHO (2002a) that have considered the potential relationship between non-occupational exposure to DDT and female breast cancer, the great majority of these studies (26) showed no relationship between DDT and breast cancer risk. Two studies showed a positive association (WHO, 2002a). In studies on general populations exposed to low levels of PCBs, none have detected a statistically significant increase in breast cancer risk, although interpretation of the data is difficult because PCBs are a mixture of congeners, containing both estrogenic and antiestrogenic compounds. Overall, the data do not support an association between PCB exposure and increased breast cancer risk.

There are limited data on the association between breast cancer and exposure to dieldrin. A study by Hoyer *et al.,* (1998) including 268 cases from a cohort of 7,712 Danish women found a two fold increase in the risk of breast cancer associated with the highest plasma concentration of dieldrin. This is an association only with no evidence of an endocrine mechanism.

Since development of the mammary gland occurs at distinct stages including perinatal, puberty and pregnancy, the timing of exposure may be critical to defining the dose-response relationships of xenoestrogens for breast cancer. The importance of timing of exposure is supported by human data on radiation and smoking and from animal studies (Tokunaga *et al*., 1987, Palmer *et al.,* 1991, Colerangle and Roy, 1997) . Adult women currently at risk for breast cancer may have been exposed to exogenous xenoestrogens (such as DDT) *in utero* or during infancy, childhood and adolescence when contaminant levels of organochlorines were higher.

Overall, the current scientific evidence, from both human and animal studies do not support a direct association between exposure to xenoestrogens and increased risk of breast cancer (WHO, 2002a). However, all these studies have measured xenoestrogen exposure in adult women and have considered exposure to individual xenoestrogens. Breast cancer is most likely multifactorial, including genetics, lifestyle, diet, endogenous hormone status and environmental factors, including timing of exposure. The interaction between these factors is unexplored and the role of xenoestrogens in breast cancer remains controversial.

Endometrial

Because endometrial tissue is very responsive to the actions of antiestrogenic and estrogenic compounds, it is a candidate target tissue for xenoestrogens. Epidemiologic data on the effects of environmental EDCs on endometrial cancer are limited. Sturgeon *et al.,* (1998) found no association between endometrial cancer and PCBs and organochlorine compounds. Hiroi *et al.,* (2004) reported lower levels of estrogenic BPA in the serum of patients with complex endometrial hyperplasia and endometrial cancer compared with healthy controls or patients with simple endometrial hyperplasia. This result does not support estrogenic compounds as causative agents for endometrial cancer. The lower prevalence of endometrial cancer in Japanese and US (Hawaiian) women consuming isoflavone rich diets supports a protective role of phytoestrogens in endometrial cancer (WHO, 2002a). Thus, there is currently no evidence to support an association between exposure to xenoestrogens and risk of endometrial cancer.

Prostate

A comparison of prostate cancer incidence rates between migrants and residents of the country of origin supports a role of environmental or lifestyle factors in prostate cancer incidence (Doll and Peto, 1981). Little is known about the causes of prostate cancer but it is both hormone dependent and able to be modulated by hormone treatment (WHO, 2002 a, Ho, 2004). Epidemiological studies undertaken in the USA (Settimi *et al.,* 2003) and Italy (Alavanja *et al.,* 2003) show an increased risk of prostate cancer with exposure to organochlorine pesticides. Animal studies are limited by the lack of suitable models (Bosland, 1992). Human prostate cells contain ERs such that cancer may be the result of cell proliferation via an ER response (Ho, 2004). It seems highly probable that estrogenic

compounds have some role in prostate cancer since it is known that expression of one ER subtype changes as prostate cancer progresses (Latil *et al.,* 2001, Lau *et al.,* 2000). Further detail is provided in Chapter 7.1.1.

Testicular

Cryptorchidism is a known risk factor for testicular cancer, suggestive of a possible prenatal etiology for this cancer (Moss *et al*., 1986). The implication is that if environmental compounds are causative agents then timing of exposure (i.e. *in utero*) may be important. Limited data suggests that the incidence of cryptorchidism and hypospadias may show similar geographical differences to the incidence of testicular cancer, suggesting that these conditions may be linked (WHO, 2002a). Altered expression of ERs between human normal and testicular tissue, implicates a role for ERs, and hence estrogenic compounds, in testicular cancer (see Chapter 7.1.2) (Hirvonen-Santti *et al.,* 2003, Pais *et al.,*2003). Studies of germ cell testicular cancer are hampered by the lack of suitable animal models. Overall, estrogenic compounds cannot be eliminated as having a role in testicular cancer but the supporting evidence is currently weak.

Thyroid

The thyroid gland has a key role in endocrine, metabolic and physiological functions. Thyroid hormones are important for growth and development and are involved in the carcinogenesis process by affecting tumor formation, growth and metastasis (Guernsey and Fisher, 1990). Thyroid cancer is uncommon, relative to other forms of cancer (NZHIS, 2004) and the etiology is largely unknown. A direct association between exposure to specific xenoestrogens and thyroid cancer is not supported by human experimental data. However, environmental chemicals including pesticides and PCBs can affect the hypothalamic-pituitary-thyroid axis. The link between this and thyroid cancer needs clarification (WHO, 2002a).

In addition to reproductive, central nervous, immune system and hormone responsive cancers, interference with steroid biosynthesis and metabolism is another source of estrogen-mediated adverse health effects (Sanderson and van den Berg (2003). Of particular interest is the inhibition of aromatase, the enzyme that catalyzes the conversion of androgens to estrogens. Possible aromatase inhibitors are some pesticides (including

DDT and its metabolites), organotin compounds, dioxins and PCBs. The consequences of such inhibition are largely unknown at this time.

Both the global WHO (2002a) and IUPAC/SCOPE (2003) reviews conclude that after substantial research in the past decade, there have been no conclusive findings of low-level environmental exposures to xenoestrogens causing human disease i.e. no proven cause and effect relationships. There is little evidence to link xenoestrogen exposure with spontaneous abortion, cryptorchidism, hypospadias and immune function. However there is more experimental and epidemiological evidence together with biological plausibility (i.e. via a possible estrogen receptor mechanism) for xenoestrogens to have a potentially causative role in sperm quality, infertility, spontaneous abortion, sex ratios, precocious puberty, neurobehaviour, breast, endometrial, prostate and testicular cancer. Food is a major route of exposure to environmental chemicals and humans are exposed to complex mixtures from the diet as a whole rather than to single compounds. Surely the total burden of xenoestrogens needs to be considered in the study of links between human exposure and health.

1.3.2.5 Positive effects

Some naturally occurring xenoestrogens (e.g. phytoestrogens) are actively promoted in functional foods, sold as dietary supplements, and as an alternative to hormone replacement therapy because of their claimed health benefits. They have been claimed to be beneficial in (COT, 2003):

- Cardiovascular disease
- Hormone-dependent cancers, particularly breast cancer
- Cholesterol-related disorders
- Postmenopausal conditions such as osteroporosis
- Stomach disorders
- Cognitive functions

A good example is a brand of bread sold in Australasia that proudly advertises its soy and linseed content (soy and linseed are rich in the phytoestrogens genistein and coumestrol respectively) and its positive effects on women's health. Whether this and related claims are true is uncertain because there are no good, controlled clinical studies that substantiate

the claim. Despite this it is important to remember that xenoestrogens (i.e. phytoestrogens) in food might be beneficial in some circumstances.

1.4 Mechanisms of action of xenoestrogens

EDCs can mitigate adverse effects by a number of different mechanisms (WHO, 2002a, SCOPE/IUPAC, 2003). One xenoestrogen may affect multiple target cells by multiple mechanisms. The mode of action may be by :

- Activating or suppressing expression of the androgen receptor
- Activating or suppressing expression of the ER, via a genomic or non-genomic pathway
- Activating the aryl receptor
- Inhibition of steroid hormone synthesis, transport or metabolism
- Modulation of neurotransmitter receptors
- Other less defined mechanisms

The selection of xenoestrogens for consideration in this thesis is restricted to chemicals in the diet that are known to mediate a potential effect via activating or suppressing expression of the ER.

ERs are nuclear receptors present in many cell types. They are large protein molecules with a specific binding site that has conformational specificity for the estrogen molecule (i.e. in a natural context 17β-estradiol). Despite the specificity of the binding site, it is sufficiently generic to allow binding by a wide range of natural and synthetic compounds. Once an estrogenic compound has diffused into a cell nucleus, it may bind to the ligand-binding domain of the receptor, whence the ER-ligand undergoes a conformational change, and dimerizes. The dimers, with cofactors, form a complex that binds to specific sequences of DNA, known as estrogen-response elements (ERE), of estrogen responsive genes. Binding to the ERE stimulates transcription and mRNA synthesis leading to increased or decreased expression of estrogen responsive genes that code specific protein syntheses (Gruber *et al*., 2002, WHO 2002a). This is the classic pathway of estrogenicity resulting in an overall effect of feminisation at a cellular and whole body level and is shown schematically in Figure 1.4.

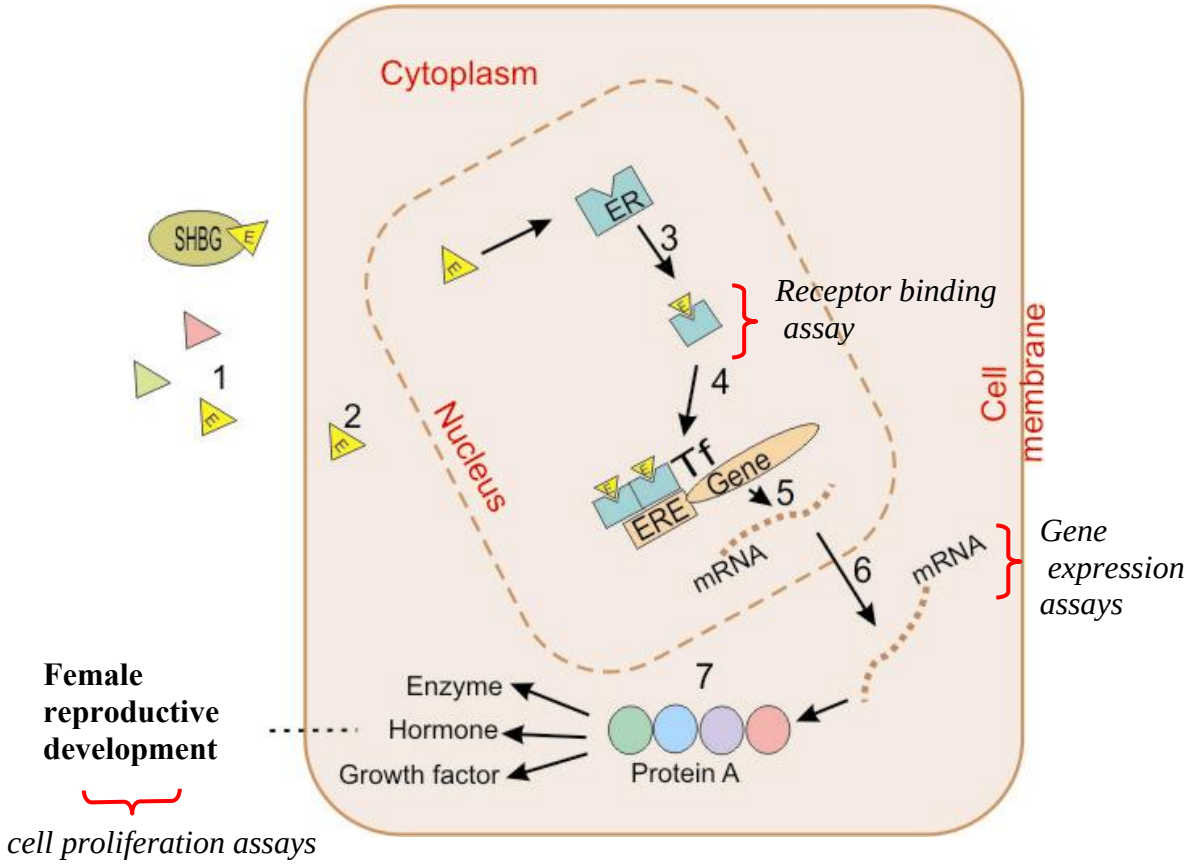


Figure 1.4: Schematic of (xeno)estrogen nuclear receptor mediated mechanism (modified from WHO, 2002a)

1. Endogenous estrogen or xenoestrogens circulating in the plasma, may be bound to SHBG. 2. Estrogenic compound diffuses into the cell and binds to ER located in the nucleus (3). 4. Occupied receptor undergoes conformational change, dimerises and binds with cofactors (Tf) to specific sequences on estrogen responsive genes. 5. mRNA is transcribed. 6. mRNA is transported from the nucleus to the cytoplasm. 7. Proteins are synthesized from the mRNA template.

There are alternative mechanisms for estrogenicity. The ERs can be also be activated independently of ligand binding (e.g. by growth factors) and estrogens can stimulate a rapid nonnuclear effect (e.g. via the mitogen-activated protein kinase pathway) (Gruber *et al.*, 2002a). However, this thesis focuses on the classic pathway of ligand-dependent activation of the nuclear ERs.

There are at least two subtypes of ER, namely α and β. Although most xenoestrogens tested bind with similar affinity to both subtypes, differences in the amino acid sequences means that some ligands bind with different affinities to the two receptor subtypes (Table

1.1). Notably, the phytoestrogens have been shown to have a greater binding affinity for ERβ.

Table 1.1: Relative binding affinities (the higher the number the greater the binding affinity) of different estrogens and xenoestrogens for the α- and β-ERs (Gruber *et al.*, 2002).

Estrogen/Xenoestrogen	ER	
	α	**β**
17β-Estradiol	100	100
Estriol	14	21
Estrone	60	37
4-Hydroxyestradiol	13	7
2-Hydroxyestrone	2	0.2
Genistein	4	87
Coumestrol	20	140
Daidzein	0.1	0.5
4-Octylphenol	0.02	0.07
Nonylphenol	0.05	0.09

Tissue distributions or expressions of ER α and β differ although there is some overlap. Granulosa, developing spermatids, intestinal mucosa, lung parenchyma, bone, brain endothelial, prostate, bladder and cells contain mostly receptor β. Endometrium, kidney, pituitary, epididymis, adrenal, breast-cancer and avrian stroma contain, or express, mostly ER α (Kuiper *et al.*,1997, Gruber *et al*., 2002). In contrast testis, uterus and ovary cells express both α and β receptors.

For both ER α and ERβ, the conformation of the ligand binding domain changes in different ways for different ligands and therefore a ligand binding to an ER α may elicit a different response than when the same ligand binds to ERβ, thus resulting in tissue specific responses (Gruber *et al*., 2002).

1.5 Structure activity

Xenoestrogens include a number of classes of compounds that look structurally different (Figure 1.5) yet can bind to the ER and elicit an estrogen-like response.

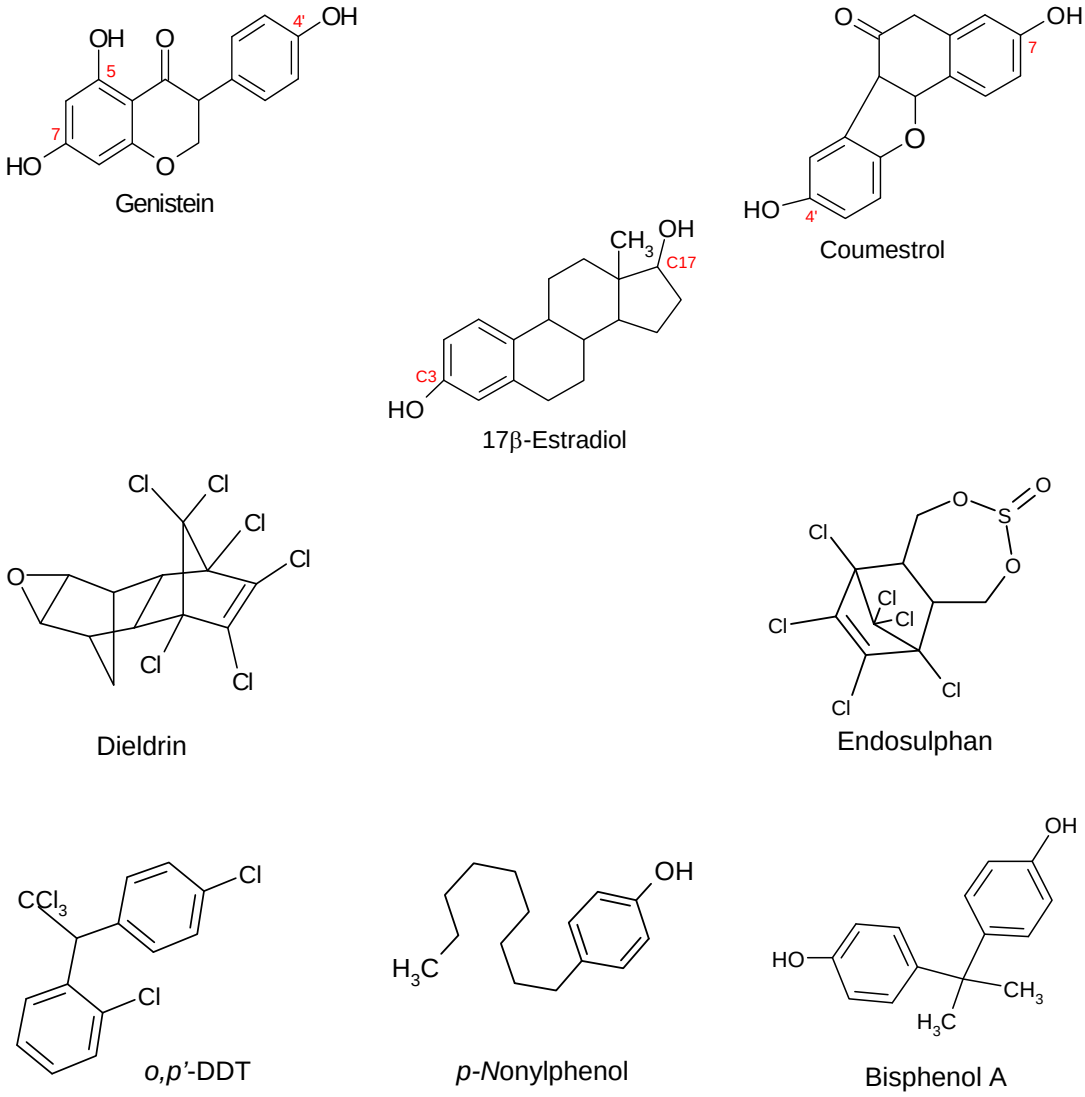


Figure 1.5: Chemical structures of a selection of estrogenic compounds including, for comparison, the endogenous hormone 17β-estradiol.

The ligand-binding domain of the ER contains amino acids that interact on a conformationally specific basis with particular chemical groups on the estrogen molecule (Figure 1.6). For example, there is a requirement for two electronegative groups a specific distance apart (they probably hydrogen bond to amino acid residues in the binding site) and a hydrophobic region between the electronegative groups (which is likely to interact with hydrophobic amino acids in the binding site by Van der Waals forces). 17β-Estradiol is the best fit for the ER and therefore results in a maximum response. Other molecules that have structural similarities to 17β-estradiol will also fit the site, but less well and so illicit a diminished ER-mediated response.

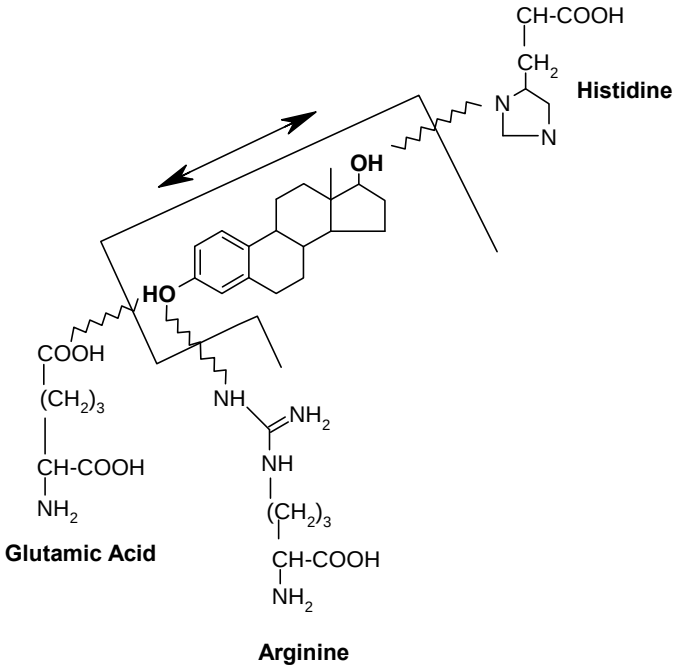


Figure 1.6: Interaction between ER amino acids and 17 β-estradiol in the ER active site

Structure activity relationships (SARs) have been elegantly explored by Fang *et al.*, (2001). They considered 230 chemicals including both naturally occurring and synthetic xenoestrogens. Activity was based on a validated ER competitive binding assay over a 10^6-fold range. They identified the following five distinguishing criteria as being essential for xenoestrogen activity:

- H-bonding ability
- the distance between the 3-and 17β-OH
- a rigid structure
- steric moieties at 7α and 11β-positions
- satisfactory hydrophobicity

If a chemical contains a phenolic ring and any additional key structural features it tends to be an ER ligand. Whilst it is useful to explore SARs as a basis for predicting estrogenicity (e.g. for drug design), the SARs proposed by Fang and co-workers leave some thoughts to ponder. For example, why is the *o,p'*- isomer of DDT more estrogenic than the *p,p'*-DDT isomer?

1.6 Assays for estrogenicity

Xenoestrogens have different degrees of estrogenicity. For example, DDT has 10^{-6} of the activity of 17β-estradiol (the natural ER agonist) (Soto *et al*., 1995) which must be taken into account when determining exposure levels, since it is estrogenicity not concentration that is important in pharmacological effect (Shaw and McCully, 2002).

A range of *in vivo* and *in vitro* assays are available to measure estrogenicity.

1.6.1 *In vivo* assays

The best assays for assessing estrogenic effects are those that use target species responses because they allow for interactions between cells and between different components of the endocrine system and thence the whole animal. In addition, they take account of absorption, disposition, metabolism and excretion of test chemicals. Assays usually employ immature, ovariectomised adult or male animals to minimise contributions from endogenous estrogens. A diverse array of assays have been reported in the literature, including:

- Measures of cell proliferation in the rodent female genital tract. Examples include; measurement of uterine epithelial height and vaginal epithelial thickness in ovariectomised mice (Ulrich *et al*, 2000), measurement of uterine weight in weanling mice (Shelby *et al*, 1996).
- Measures of other estrogen related effects on the female genital tract, including increases in uterine vascular permeability (Milligan *et al*, 1998), increases in glycogen production in immature rat uteri (Bitman and Cecil, 1970).
- Measures of testicular changes or sperm quality in male rodents, including reduction in testicular size, reduction in ventral prostate weight, and reduction in sperm production in male rats (Sharpe *et al*, 1995).
- Induction of vitellogenin in fish. Induction of the protein, vitellogenin, in male and female fish after exposure to estrogenic compounds has been carried out for a number of fish species, including; rainbow trout (Sumpter, 1985), carp (Tyler and Sumpter, 1990), and winter flounder (Pereira *et al*, 1992).
- Sex determination in turtles. The sex of offspring of the red-eared slider turtle, as for other reptiles, is determined by the temperature of incubation. The ability of

xenoestrogens applied to the eggshell to overcome this effect was used as an assay of estrogenic activity (Bergeron *et al*, 1994).

For most human dietary xenoestrogens, this data is lacking.

1.6.2 *In vitro* assays

In vitro estrogen assays fall into three groups corresponding to steps in the ER-dependent metabolic pathway that they assay (see Figure 1.4). The groups are:

- Receptor binding assays, which measure the strength of the interaction between the ER and the estrogenic compound.
- Receptor-dependent gene expression assays, which measure the ability of the estrogenic compound to activate cells' biochemical responses, via the ER and hence to produce a protein or proteins.
- Cell proliferation assays, which measure an estrogen-dependent cellular response.

1.6.2.1 Receptor binding assays

These assays measure the strength of the interaction between the ER and the test xenoestrogen in a competitive binding assay with 17β-estradiol. Assays utilise ERs from mouse uteri (Korach *et al.*, 1988, Krishnan *et al.*, 1993), spotted seatrout liver (Thomas and Smith, 1993), rainbow trout liver (White *et al.*, 1994), cultured MCF-7 human breast cancer cells (Nagle *et al.*, 1997) and cultured ECC-1 human endometrial carcinoma cell lines (Bergeron *et al.*, 1999).

1.6.2.2 Receptor-dependent gene expression assays

Receptor-dependent gene expression assays measure the ability of the estrogenic compound to cause a conformational change in the receptor leading to DNA transcription and gene expression. Examples of assays of this type include:

- the yeast estrogen screen (YES), created by transfecting yeast cells with the human ER and two estrogen response elements linked to the *lacZ* reporter gene, coding for β-galactosidase (Arnold *et al.*, 1996) which is upregulated and leads to the conversion of a yellow galactoside dye to red chromophore (plus galactose) that can be measured spectrophotometrically.

- HeLa cells, which do not contain an ER, co-transfected with an estrogen responsive reporter vector (ERET81CAT) and a mouse ER vector (pRSV). Estrogenic activation results in production of chloramphenicol acetyltransferase which is detected by ELISA (Shelby *et al.*, 1996).
- the production of cathepsin-D and pS2 from MCF-7 human breast cancer cell line can be monitored with the ELSA-CATH-D and ELSA-PS2 immunoradiometric assays following exposure to estrogenic compounds (Olea *et al.*, 1996).
- production of complement C3, a well known estradiol-regulated protein from a cultured rat endometrial adenocarcinoma cell line (RUCA-I). Proteins are labelled with [^{35}S]-methionine and determined by autoradiography after electrophoretic separation (Hopert *et al.*, 1998).
- production of zona radiata proteins and vitellogenin in primary hepatocytes from Atlantic salmon exposed to xenoestrogens *in vitro.* Protein products are determined by ELISA (Celius *et al.*, 1999).

1.6.2.3 Cell proliferation assays

Cell proliferation assays measure an estrogen-dependent physiological response resulting from the production of functional proteins. The ability of chemicals to induce ER positive cell proliferation is an indicator of estrogenic activity (Soto *et al.*, 1995). Assays of this sort can be carried out *in vitro* by utilising established cell lines derived from estrogen responsive target organs, such as rat pituitary cells and human breast cancer cell lines (T47-D and MCF-7). MCF-7 is by far the most commonly used cell line for assays of estrogenicity and is the basis for the so-called E-Screen assay (Soto *et al.*, 1995).

1.6.3 Choice of assay and comparative results from different assays using EQ

The yeast (YES) and E-Screen assays are the most widely used. Whilst the majority of assays have employed purified individual test chemicals and the response compared to a reference compound (generally 17β-estradiol), the yeast assay has been used to study the effect of mixtures and to measure estrogenicity of foodstuffs (Silvia *et al*., 2002, Takamura-Enya *et al.,* 2003).

Results from the different assay methods cannot be compared directly. However it is arguable that relative estrogenicity (i.e. relative to 17β-estradiol) can be compared

between assays. The 17β-estradiol equivalence factor (XEQ) is the ratio of the concentration of the xenoestrogen to the concentration of 17β-estradiol that produced the same response in an estrogenicity assay. Rudel (1997) has reviewed the use of XEQs (that he termed 'TEFs' (Toxic Equivalent Factor)) in assessing risk from xenoestrogens and states that the use of XEQs is reliant on two assumptions:

- that the relative xenoestrogenic potency in *in vitro* assays for all compounds is predictive of the relative potency for the most sensitive *in vivo* xenoestrogenic effect, and
- that all xenoestrogens act via the same mechanism.

He provides significant evidence that different xenoestrogens may act by different mechanisms to produce a multitude of different effects, and that the use of XEQs represents a significant simplification of this complex and poorly understood system. The TEF approach is adopted for assessing overall effect of exposure to dioxins (van den Berg *et al.,* 2000) and it is the author's view that this simplification is a reasonable basis for relative potency for xenoestrogens that act by the same mechanism (such as via the nuclear receptor pathway) to provide an estimate of risk and to suggest priorities for further risk analysis.

1.7 Additivity of xenoestrogens

A significance component of this thesis is to assess the cumulative risk to public health due to the exposure of the population to a diverse array of xenoestrogenic compounds through the diet. The assessment of chemical mixtures is a complex topic for toxicologists, regulators and the public (Monosson, 2005). Such a cumulative approach can only be undertaken if a reasonable model can be applied to combine the xenoestrogenic effects of different groups of xenoestrogenic compounds. The effect of mixtures can be classified as synergistic (greater than expected), antagonistic (less than expected), or additive (as expected). In the absence of evidence of chemical interaction, assumption of no interaction is the default approach (USEPA, 2000).

While some studies have suggested that xenoestrogenic compounds may combine their effects in a synergistic manner (Arnold *et al.*, 1996), this finding has not been reproduced in subsequent studies (Gaido *et al.*, 1997; Kortenkamp and Altenburger, 1999; Payne *et*

al., 2000). Gaido *et al.,* (1997) reported additivity of effect for combinations of organochlorine compounds. Payne *et al.,* (2000) demonstrated that the effect of four xenoestrogens (*o,p*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol) in a yeast estrogen screen (YES) could be predicted using a model of concentration addition, when due consideration was given to the characteristics of the individual dose-response curves.

Clear evidence of additivity of effect has been demonstrated by Kortenkamp and colleagues (Payne *et al.,* 2000, Silva *et al.*, 2002, Rajapakse et al., 2002) who tested the estrogenicity of xenoestrogen mixtures including alkyl phenols, hydroxylated PCBs, benzophenones, parabenes, BPA and genistein using the yeast assay. Compounds were combined at concentrations below individual No Observable Effect Levels in proportion to their individual potencies. When combined, an estrogenic response was measureable. The response of the compounds in combination showed good agreement with the estimated response of the individual xenoestrogens, demonstrating that the combined effect was additive. In subsequent work, additivity was less than estimated when a similar experiment was undertaken using the MCF-7 cell proliferation assay (Rajapakse *et al*., 2004). A different suite of estrogenic compounds were used than for the yeast assay experiments. The lack of additivity in this case was ascribed to two alkyl phenols that were estrogen antagonists when present in a mixture. Possible interactions between components of a mixture resulting in deviation from additivity must be kept in mind when assessing the effect of combinations of xenoestrogens in the diet.

Using molecular techniques to measure gene expression Gaido *et al.,* (2003) found that endocrine effects of a selected chemical were tissue and mixture dependent meaning that EDCs could be additive for one response and antagonist for another response. When estradiol and HPTE (an hydroxylated analogue of DDT) were added individually to the reproductive tissue of male and female mice, they were found to act similarly on expression of most genes in the ovaries, uterus, testes and prostate. However, for each tissue type there were subsets of genes differentially regulated by the 2 compounds. When added in combination, the effect was often, but not always additive (e.g. expression of IGF binding protein in the testis). Similarly genistein and methoxychlor showed an additive effect on vaginal opening and altered estrous cyclicity in female Sprague-Dawley rats but genistein enhanced the effects of methoxychlor on preputial separation in male rats.

For the purpose of the current risk assessment a concentration addition model is applied as a basis for assessing a maximum possible effect. While this is clearly a simplification, it represents the most pragmatic approach based on the current state of knowledge, is generally supported by research on this topic and errs on the side of safety for deciding whether these compounds might be of concern for human health.

1.8 Risk assessment

If a person (or wildlife species) is not exposed to a hazard, then they will suffer no adverse effects as a result of that hazard, there is no risk to them. Risk assessment is a scientifically based process, invaluable for defining the importance of any particular hazard. The model of risk assessment described by the National Research Council of the National Academy of Sciences (NAS-NRC, 1983), and used by government agencies including the Environment Protection Agency (EPA), consists of :

- hazard identification
- dose-response
- exposure assessment and
- risk characterization.

Hazard identification

Hazard identification relates to whether a specified chemical causes a particular health effect. In the context of this thesis, hazard identification relates to the selection of dietary constituents that might contribute to total estrogenicity from the diet. Ideally, all chemicals in food with estrogenic activity would be included. However, since different xenoestrogens have different estrogenic potencies, a XEQ approach is necessary to allow for the differences in potency between xenoestrogens. A XEQ approach requires a common mechanism of action. Given that the main (or classical) action of xenoestrogens is via the nuclear ER, it is appropriate to base hazard selection on xenoestrogens that have been shown to act via this pathway. On this basis, compounds that activate or suppress expression of the androgen receptor, inhibit steroid hormone synthesis, activate the aryl receptor, or modulate neurotransmitter receptors would not be included. Thus genistein

would be included but phthalates and dioxins that act via binding to the aryl receptor, would not.

Dose-response

Dose-response is a measure of the relationship between the magnitude of exposure and the probability of health effects (NAS-NRC, 1983). For xenoestrogens in the diet this translates to how much xenoestrogen gives a measureable estrogenic effect, where effect can be measured in a variety of ways including human epidemiological studies, animal studies, *in vivo* or *in vitro* assays (see 1.6). Ideally, a whole organism effect would be the basis for dose –response data but in the absence of this, a common *in vitro* assay basis is preferred because different assays can give different relative responses to different xenoestrogens (for example see Chapter 2.7.1 relating to DDT). For xenoestrogens, effect based on *in vitro* cell proliferation assays is considered the best option for two reasons. Firstly, this assay represents a higher level of biological complexity than either competitive binding or gene expression assays. Secondly, cell proliferation assays based on the MCF-7 breast cancer cell line are one of the most widely employed asssays of estrogenicity providing a common basis for diverse xenoestrogens (Soto *et al.,*1995, Breinholt and Larsen, 1998).

Exposure assessment

Exposure assessment is the measure of the amount of hazard to which a person or organism is exposed, or the likelihood of being exposed. For a foodborne hazard, exposure is equivalent to intake where intake is the product of the concentration of the hazard in the food and consumption of that food item. For example, the dietary intake of a pesticide residue in a given food is obtained by multiplying the residue level in the food by the amount of that food consumed (WHO, 1997). Food consumption may be determined in a number of ways (WHO, 1997):

i. Per capita consumption from food balance sheets that estimate average food consumption from national data of food production, less exports, plus imports, minus wastage. Food balance sheets are thought to overestimate consumption of most commodities and therefore represent high percentile consumers.

i. Household budget data, that is often a refinement of (i) above as they reflect food purchased for human consumption.

ii. Food diary records, where respondents record all food consumed over a specified period.

iii. 24-hour diet recalls, where a sample of respondents recall all food consumed in a defined 24 hour period.

iv. Food frequency questionnaires where respondents recall how often a food is consumed.

Risk characterization

Risk characterization brings together dose-response and exposure to estimate the nature and magnitude of human risk, including uncertainty. For example, if a person does not consume canned food, their risk of feminizing effects from BPA that might be present in the canned food, will be zero. For xenoestrogens, if dose-response is based on cell proliferation effects, then the derived risk characterization would describe the magnitude of cell proliferation for the derived level of exposure. But in terms of human health, this is not very descriptive because cell proliferation is not the only likely (adverse) outcome of exposure. The pharmacological impact of xenoestrogens is dependent on the blood level attained relative to normal levels of endogenous estrogens. Therefore if a combined blood concentration of exogenous estrogenicity was either a significant proportion of, or greater than, the endogenous estrogenicity, it is highly plausible that a pharmacological effect will result. Since in a XEQ approach, xenoestrogen effects are standardized relative to the endogenous hormone 17β-estradiol, it is reasonable to assess risk from exposure to xenoestrogens as a proportion of normal circulating levels of 17β-estradiol, as followed by Shaw and McCully (2002). This approach assumes that the transfer from blood across cell membranes to target receptors is the same for endogenous estrogen as for xenoestrogens.

1.9 Work described in this thesis

The background information provided in the introductory Sections 1.1 through 1.7 demonstrates an international interest in estrogenic compounds. Evidence for the effects on wildlife are more convincing than the array of associated human health effects, both positive and negative. Causal links between estrogenic hazards and human health need to be substantiated. This is difficult given the usually long latency between exposure and observed effect. In assessing the hypothesis that dietary xenoestrogens may contribute to adverse endocrine effects, the thesis addresses the overarching question:

"Is the level of exposure to estrogenicity from dietary constituents in combination sufficient (in the context of normal body levels of estrogens) to have a pharmacological effect?"

A comprehensive risk assessment of naturally occurring and synthetic estrogenic compounds found in food is presented (Chapter 2). Several contributing questions are subsequently addressed from data gaps identified from this risk assessment:

- What is the level of exposure to BPA in the New Zealand diet?
- Do microflora account for low plasma levels of isoflavones compared with intake levels?
- What is the maternal to fetal transfer of xenoestrogens?

This exposure assessment combines new and existing data on food concentration, food consumption and serum levels for each xenoestrogen. Exposure is combined with relative estrogenic potency information, from published bioassay data, to estimate risk relative to normal circulating levels. Four areas that are identified from the **risk assessment** are then pursued from a multidisciplinary approach.

A priority data gap, namely the quantitation of BPA in canned food and subsequent population exposures are described in Chapter 3, drawing on **quantitative analytical chemistry** using gas chromatography mass spectrometry with an isotopic internal standard.

Human intestinal microflora are reviewed and **microbiological techniques** applied in combination with high-pressure liquid chromatography to study the role of gut microflora on the bioavailability of the phytoestrogens genistein and daidzein in Chapter 4.

A "gene expression" **biological assay** for measuring estrogenic potency is validated in Chapter 5. The assay, utilising a yeast strain incorporating the human ER is used in experiments to explore ***in utero*** exposure to estrogenic compounds. The dual perfusion placental model is further applied to measure the transfer of xenoestrogens across the human placenta in Chapter 6.

The thesis concludes with preliminary experiments towards a genomic approach to investigate exposure to xenoestrogens with a biochemical response in human cell lines, Chapter 7.

Chapter 2

Risk assessment of dietary exposure to xenoestrogens

2.0 Introduction

Food is a major source of exposure to xenoestrogens. A number of investigators have assessed the exposure of selected populations to individual groups of xenoestrogens (eg Maskarinec *et al.,* 1998, Arai *et al*., 2000a,b, Hertog and Hollman 1996, CAC 2000, Hardy 1998, Gunderson 1995, Howe *et al.,* 1998, Botterweck *et al.,*2000). However, there are only two reports describing the relative contribution of different groups of xenoestrogens to total estrogenic intake from the diet (Safe 1995, Shaw and McCully, 2002). Safe *et al.,* compared the estrogenic intake of flavonoids in foods with the estrogenicity of four combined pesticides using a combination of binding affinity and cell proliferation respectively. No evaluation was attempted of the pharmacological significance of this level of dietary exposure to xenoestrogens. Shaw and McCully (2002) estimated the estrogenic intake of several dietary xenoestrogens in terms of 17β-estradiol equivalents as determined by a cell proliferation assay. Included in the comparison were coumesterol, genistein, bisphenol-A, phthalates, DDT, dicofol, endosulphan, dieldrin and β-HCH. A theoretical blood serum concentration was calculated, assuming 100 percent absorption, and compared with circulating levels of 17β-estradiol. This is a simplification since 100% absorption does not occur (Xu *et al.,* 1995, Arai *et al.,* 2000a). Both the Safe and Shaw studies showed that the contribution of synthetic compounds to the intake of estrogenicity was low relative to the estrogenicity from naturally occurring xenoestrogens.

However, a more comprehensive estimate is now possible compared to those reported by Safe (1995) and Shaw and McCully (2002). A wider range of xenoestrogens, more robust concentration and estrogenicity data, and allowance for factors influencing serum levels can be considered. In this chapter, dietary exposures to a total of 13 groups of estrogenic compounds are assessed from food concentration, and food consumption information for a sample of New Zealand adults.

2.1 Rationale for risk assessment

2.1.1 Selection of chemicals

Chemical compounds for the exposure assessment were included on the basis of consideration of the scientific literature. Compounds, or closely related groups of compounds, were included in the current assessment if:

- There was consistent evidence of the chemical having xenoestrogenic activity, either *in vitro* or *in vivo* and that the chemical's mechanism of action was via the ER. Evidence of a common mode of action was considered to be a necessary criterion for xenoestrogenicity for validity of an XEQ approach for different chemicals to be considered in aggregation (Rudel, 1997, van den Berg *et al*, 2000).
- There was evidence for their presence in the New Zealand diet. For instance, pesticides that have never been registered for use in New Zealand and have never been reported to be detected in foods in New Zealand were not included in the intake assessment.

Assessment based on the above criteria resulted in inclusion of the four specific compounds and nine chemical classes (Table 2.1.1).

Table 2.1.1 Xenoestrogens included for dietary assessment

Natural compounds	Synthetic compounds
Isoflavones	DDT and its metabolites
Lignans	Aldrin and Dieldrin
Coumestans	Endosulfan
Flavonoids	Synthetic pyrethroid pesticides
Resorcylic lactones	PCBs
	Alkyl phenols
	Bisphenol-A
	Butylated Hydroxyanisole (BHA)

2.1.2 Concentration data

New Zealand data were used whenever they were available for population consistency with consumption data. Median concentration is desirable for intake estimates, to reflect the intake of the majority of consumers, but in the absence of this data, mean levels of chemicals in foods were used for exposure estimates (WHO, 2000). Where concentration data was available only as a range (for the isoflavones, coumestrol, quercetin and phloretin), both upper and lower intake estimates were determined. Where New Zealand data were not available or do not exist (such as nonyl phenol), data from the world scientific literature were employed.

2.1.3 Food consumption

The two primary sources of food consumption information used to derive exposure estimates for xenoestrogens in the New Zealand diet were:

- 1997/98 New Zealand Total Diet Survey simulated typical diets (Cressey *et al.,* 2000). These diets were based largely on national consumption data (Horwath *et al*., 1991) with input from other sources of information (e.g. fish industry data). The vegetarian simulated diet was based on two New Zealand studies (Alexander *et al.*, 1994, Harman and Parnell, 1998). Simulated diet information was used as much as possible as it is designed for population sub-group dietary intake estimates. However, the number of foods included limits the usefulness. Food consumption for the simulated diets are included in Appendix 2.
- The 1997 National Nutrition Survey (NNS) (Russell *et al.,* 1999) was used for specific foods not included in simulated diets (for example, isoliquiritigen in licorice, and BPA in canned food). Further detail is given in 8.2.1.

For the foods not reported in the National Nutrition Survey (and therefore not consumed by any of the 4636 respondents), an assumption was made that one person in 5000 would consume 50 g of these foods on a daily basis. This equates to a daily level of consumption for the average New Zealand resident of 0.01 g/day. This approach was used to estimate intake of coumestrol from split peas, kala chana seeds, lima and pinto beans and soyabean sprouts.

2.1.4 Population sub-groups

Exposure assessments were undertaken for the following sub-populations;

- an average adult male (≥ 25 years), mean body weight 80kg
- an average adult female (≥ 25 years), mean body weight 65kg
- a 19-24 year old male, mean body weight 70kg
- a lacto-ovo vegetarian (19-40 years) (no animal products other than dairy and eggs), mean body weight 65kg

as representative of the majority of the population and because food consumption data for these groups could be accessed from both the simulated model diets and the NNS. The 19-24 latter group was included because this is the population sub-group with the highest average level of food consumption, thence a high potential exposure. The lacto-ovo vegetarian female was included as a group potentially at risk because of dependence on a narrower range of foods than the general population. Exposure to younger people was excluded because of the lack of rigour in consumption information and not because of a low risk for this sub-population group. Food consumption data for vegetarian females, that may be an at risk sub-group, was available from the simulated diets but not NNS data. Where consumption information was not available (coumestrol, isoliquiritegenin and bisphenol A), exposure for this sub population was assumed to be the same as for adult females.

2.1.5 Relative estrogenic potency data

In the absence of sufficient data on *in vivo* effects, relative estrogenicity based on *in vitro* cell proliferation assays is considered the best option as a measure of dose-response for two reasons. Firstly, this assay represents a higher level of biological complexity than either competitive binding or gene expression assays. Secondly, cell proliferation assays based on the MCF-7 breast cancer cell line are one of the most widely employed asssays of estrogenicity providing a common basis for diverse xenoestrogens (Soto *et al.,*1995, Breinholt and Larsen, 1998). For internal consistency, results from Breinholt and Larsen (1998) were used. Where these data were not available, and where different studies derived different estimates of estrogenic potency for the same xenoestrogen, the highest estimate (worst case) was used for the risk assessment. In the absence of cell proliferation

data, the estrogenic potency of isoliquiritigenin was based on structure activity relationship to the most estrogenic flavonoid (apigenin). These worst case assumptions follow the "precautionary principle" (Burger, 2003), in recognition of the uncertainty of the potential endocrine effect.

2.1.6 Circulating concentrations of xenoestrogens

Any potential pharmacological effect of a xenoestrogen depends not on intake, but rather on the circulating concentration in the blood. A blood level, accounting for absorption, distribution, metabolism and excretion, was extrapolated from the intake estimate by adjusting for the ratio of actual (A) serum (or plasma) level to theoretical (T) serum level from published data. Information on actual serum levels used, in order of priority was:

- Studies relating dietary intake of xenoestrogens to actual steady-state levels of the xenoestrogens in serum for the same study group.
- Studies reporting serum levels of xenoestrogens for the general New Zealand population.
- Studies reporting serum levels of xenoestrogens from a region of similar diet to New Zealand.

Hence an A:T ratio was determined (see 8.2.2 for further detail). This adjustment assumes the same ratio of actual to theoretical concentrations across population groups which is undoubtedly a simplification. Nevertheless, this conversion to an internal dose is considered an improvement over previous exposure estimates.

2.1.7 Uncertainty

Quantitative or qualitative uncertainty analysis is an integral component of risk characterization or risk estimation (NAS-NRC, 1983, Covello and Merkhofer, 1996), and allows a ranking of key factors and data gaps to be prioritised. In the risk assessments that follow, there are uncertainties that are equivalent across each xenoestrogen, for example the uncertainty associated with extrapolating from a cell proliferative effect to a whole organism. In addition there are uncertainties that vary for each xenoestrogen. The following uncertainties are recognized for each component of the individual assessments that comprise the aggregated risk from dietary xenoestrogens:

- Food concentration: uncertainty arising from analytical variability, representativeness of sampling, data gaps.
- Food consumption: misreporting, errors in assigning foods, variability from combining individual consumption to population groups, data gaps.
- Estrogenic potency: analytical variability in assays, extrapolation from cell proliferation to other *in vitro* assays, extrapolation of effect from one compound to another based on structural similarity
- A:T ratio: analytical variability in plasma determinations, extrapolation from one population to another, extrapolation from NZ serum levels to NZ intake data from a different study, extrapolation from serum levels of non NZ population, no data.

Data for some xenoestrogens are more robust (less uncertain) than for other xenoestrogens. An assessment of the significance of differences between various contributing factors is guided by the following qualitative uncertainty assignments (Table 2.1.2.), where * represents least uncertainty (more robust) and *** represents the greatest uncertainty (least robust). This approach is consistent with the format proposed by Covello and Merkhofer (1996, Table 23) for qualitative uncertainty analysis where * represents minor significance and *** represents major significance.

For the current assessment, concentration data from New Zealand was rated to be more certain (*) than data from overseas (**) as it better reflects useage of industrial chemicals and food growing patterns that may influence natural chemicals for the consuming populations for which the risk assessment is being undertaken.

In nearly all cases, consumption was available for the population groups under study and an uncertainty of * was assigned. Where it was not available (e.g. alfalfa sprouts) it was assigned an uncertainty of ** to reflect the estimation.

The uncertainty of data on estrogenic potency between different xenoestrogens was ranked for analytical variability in assays from primary operator (*) to another (**), extrapolation from cell proliferation to other *in vitro* assays (***) and extrapolation of effect from one compound to another based on structural similarity (***) based on the

variabilites of potency data available for each xenoestrogen and across different xenoestrogens.

Qualitative uncertainty assignments for the A:T were ranked from the most certain scenario where intakes and serum levels were available for the same population group (*) to extrapolation of serum levels from one population to another population from the same country, and therefore assumed similar intake (**) or extrapolation from NZ serum levels to NZ intake data from a different study (**), to the extrapolation from serum levels of non NZ population (***) being the most uncertain (Table 2.1.2).

Table 2.1.2: Assignments of qualitative uncertainties

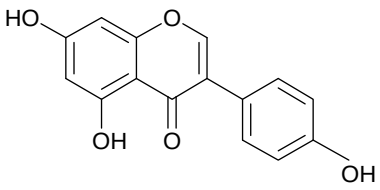
Contributing factor	Description of data source	Uncertainty assignment
Food concentration	New Zealand	*
	Overseas	**
Food consumption	New Zealand	*
	No New Zealand data	**
Estrogenic activity	Cell proliferation, Breinholt and Larsen, 1998	*
	Other cell proliferation data	**
	Other *in vitro* data	***
	Extrapolation from structure/activity relationships	***
A:T ratio	Intake and serum levels, same population, same study	*
	Intake and serum levels, same population but not the same study	**
	New Zealand serum data	**
	Other serum data	***
	Extrapolated from structure/activity relationship	***

An overall uncertainty equivalent to the maximum contributing uncertainty of any one component was assigned such that an overall uncertainty of "*" represented a less uncertain (more robust) risk assessment than an overall uncertainty of "***" in an attempt to convey what is missing, hence confidence in the risk assessment (USEPA, 2000). These assignments do not distinguish between the relative importance of estrogenic activity and A:T ratio but do mean that these components dominate over food concentration and food consumption data that have only 2 levels of uncertainty. This is a limitation of the model.

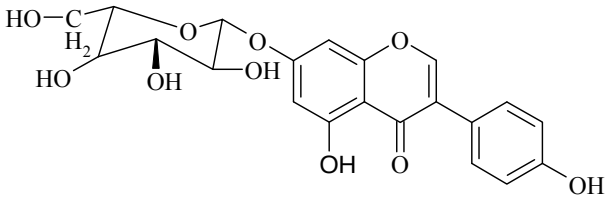
2.2 Isoflavones

Included in this group are the compounds genistein, daidzein, biochanin A and formononetin (COT, 2003). Isoflavones are often present as glucoside conjugates in plants and foods (e.g.genistin and daidzin). The glucose group may be esterified with an acetyl- or malonyl group to form acetyl- or malonylglucosides (acetylgenistin, acetyldaidzin, malonylgenistin and malonyldaidzin). Although found in many plant tissues, highest levels are observed in legumes (particularly soybeans), with more variable levels in oilseeds and nuts.

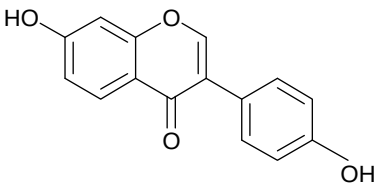
The structures of genistein, genistin, daidzein, daidzein, biochanin A and formononetin are shown below (Figure 2.1). The structural similarity of the non-conjugated (aglucone) isoflavones, to 17β-estradiol is clear with distally located hydroxyl group separated by a multiple ring structure.



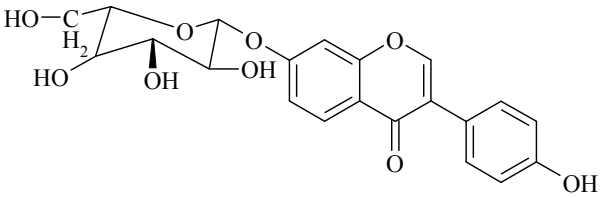
Genistein



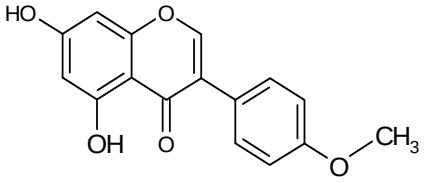
Genistin (genistein glucoside)



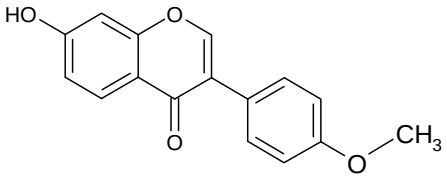
Daidzein



Daidzin (daidzein glucoside)



Biochanin A



Formononetin

Figure 2.1: Chemical structures of the isoflavones genistein, daidzein biochanin A and formononetin and the glucoside conjugates genistin and daidzein.

2.2.1 Estrogenic activity

The relative estrogenic potencies of phytoestrogens has been reviewed (IEH, 2000) including very limited *in vivo,* and more numerous *in vitro,* assays. The estrogenic potency data for the isoflavones is summarised in Table 2.2.1.

Table 2.2.1 Relative estrogenic potencies of isoflavones

	***In vitro* assays**			***In vivo* assays**
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1	1	**1**	1
genistein	0.02[1]	0.00084[4]	**0.0008**[5]	0.00001[7]
	0.001[2]	0.0001[6]	**0.0003**[8]	
	0.9[3]	0.000045[8]		
genistin		0.000048[6]		
daidzein	0.005[3]	0.00013[4]	**0.0000071**[5]	0.0000075[7]
		0.00002[6]	**0.0001**[8]	
		0.0000028[8]		
daidzin		0.000012[6]		
biochanin-A	<0.0001[3]	<0.00006[4]	**0.000048**[5]	
		0.000012[6]	**0.0001**[8]	
		0.0000025[8]		
formononetin	<0.0001[1]	0.000006[4]	**0.0000046**[5]	0.0000026[7]
	<0.0001[3]			

1 = IEH, 2000 2 = Zava *et al.*, 1997 3 = Kuiper *et al.*, 1998.
4 = Markiewicz *et al.,* 1993 5 = Welshons *et al.*, 1990 6 = Mayr *et al.*, 1992
7 = Bickoff *et al.*, 1962 8 = Breinholt and Larsen, 1998.

The values given are the highest reported where estrogenicity varied between the α and β estrogen receptor subtypes.

Relative estrogenic potencies of EQ= 0.0003 (300 x 10^{-6}) for genistein and 0.0001 (100 x 10^{-6}) for daidzein will be used for the current assessment of estrogenicity.

2.2.2 Occurrence in foods

The isoflavones genistein and its glucoside, genistin, (the sum of which is referred to as total genistein), daidzein and its glucoside, daidzin, (the sum of which is referred to as total daidzein), biochanin A and formononetin have been analysed in a range of selected foods available to the New Zealand consumer (Taylor and Burlingame, 1998). A summary of concentrations is shown in Table 2.2.2. The concentration of the glucoside conjugates, genistin and daidzin, is much higher than the aglucone forms. Since the

glucosides are readily converted to the aglucone by gut microflora, intake of both the glycoside and aglucone must be considered for a realistic exposure assessment. Concentrations of individual isoflavones greater than 15 mg/kg were limited to soy products with the exception of formononetin in red clover tablets. The concentration of the glucosides, genistin and daidzin, were higher than the parent isoflavones by factors ranging from two to two hundred. Biochanin A and formononetin were not detected in any of the New Zealand foods, with the exception of red clover tablets, which are not a food so much as a supplement.

Table 2.2.2 Concentration of isoflavones in selected New Zealand foods (mg/kg) (Taylor and Burlingame, 1998)

Food type	Genistein + Genistin	Genistein	Daidzein+ Daidzin	Daidzein	Biochanin	Formononetin
Australian soy beans	1334-1559	<2.4- 8.4	795-1059	<1.3- 7.6	<3.7	<2.0
New Zealand soybeans	611-1891	<2.4- 41	913-1419	<1.3- 29	<3.7	<2.0
Soy based infant formulas	121-201	4.6- 7.7	83.8-153.7	1.9- 24.2	<3.7	<2.0
Farex infant food	134-170	2.3- 7.1	211-394	1.3- 10.2	<3.7	<2.0
Dairy based infant formulas	<2.4-11.4	<2.4	1.5-10.8	0.2- 3.5	<3.7	<2.0
Soy flour	1765- 1801	31- 160	1151- 1281	24.1-85.4	<3.7	<2.0
Soy protein isolate	942	159	369	60.7	<3.7	<2.0
Soy chunks	916- 1570	15.5- 23.7	375- 858	17.3- 48.5	<3.7	<2.0
Soy mince	977	160	1266	175	<3.7	<2.0
Soy/TVP bacon bits	957-1613	20.9- 29.6	656- 1120	13.0- 233	<3.7	<2.0
Tofu luncheon	140	19.5	71.4	11.4	<3.7	<2.0
Soysage	50.0	9.0	4.9	4.9	<3.7	<2.0
Tempeh[1]	165	77.5	136	44.3	<3.7	<2.0
Soy yoghurt	19.7	0.7	11.9	0.8	<3.7	<2.0
Tofu	197-401	9.8-159	81.1-213	5.3-135	<3.7	<2.0
Soy milk	39.0-110	0.4-3.9	62.2-84.8	0.8-2.7	<3.7	<2.0
Miso[2]	320-671	90.0-389	184-1855	76.7-273	<3.7	<2.0
Milk	<0.5	<0.5	0.25-0.33	0.25-0.33	<3.7	<2.0
Yoghurt	<0.5-0.8	<0.5-0.8	<0.3	<0.3	<3.7	<2.0
Red clover tablets	6123	812	2162	302	<3.7	4000

1=fermented soy beans
2=soy based soup

Formononetin (0.05-4.02 mg/kg), daidzein (0.02-0.63 mg/kg), genistein (0.045-1.82 mg/kg) and biochanin A (0.23-1.38) have been measured at low levels in beer (Lapcik *et al*., 1998). Beer was not included in the foods analysed in the New Zealand study and therefore upper and lower concentrations reported in the literature were used in the exposure assessment.

A survey of the use of soy protein in New Zealand meat products was conducted in 1993 (Lake *et al*., 1993). Assuming upper and lower levels of soy protein content and mean concentrations of isoflavones in soy protein isolate reported by Taylor and Burlingame (1998), upper and lower estimates of isoflavones in these meat products can be derived. Not all of the meat products analysed contained soy protein and if it is assumed that the proportion of positives found in 1993 is representative of currently available meat products, average concentrations for each meat category may be derived as shown in Table 2.2.3.

Similarly, bread might contain soy flour as a component of bread improvers (approximately 0.5% of the flour mix). Correcting for moisture content differences between flour and bread (Athar *et al*., 1999), the soy flour content of bread would be approximately 0.35% by weight. Using the upper and lower concentrations of isoflavones in soy flour reported by Taylor and Burlingame (1998), the maximum and minimum concentrations of total genistein in bread are 6.3 and 6.2 mg/kg and total daidzein concentrations are 4.5 and 4.0 mg/kg.

Table 2.2.3 Estimated average upper concentration of isoflavones in New Zealand meat products[1]

Product category	Proportion of positive samples[2]	Range of soy protein levels (%)[2]	Genistein + Genistin Upper (mg/kg)	Genistein + Genistin Lower (mg/kg)	Daidzein+ Daidzin Upper (mg/kg)	Daidzein+ Daidzin Lower (mg/kg)
Pies (fillings)	14/34	0.5-4.5	17.5	1.9	6.8	0.8
Hams	3/10	1.5	4.2	4.2	1.7	1.7
Saveloys	4/14	0.5-2.0	5.4	1.3	2.1	0.5
Sausages	11/24	0.5-2.5	10.8	2.2	4.2	0.8
Luncheons	3/7	1.5-2.0	8.1	6.1	3.2	2.4

1 Calculated from concentration in soy protein isolate x maximum % soya protein in that food x proportion of positive samples
2 Lake *et al.,* 1993

2.2.3 Exposure assessment

Tofu, tempeh and miso are each derived from soy beans: tofu is curdled soy milk in the same way that cheese is made from milk, tempeh is made from cooked soybeans fermented with a Rhizopus mold and miso is made from fermented, salted soybeans aged

for 1-3 years. Very little tofu, tempeh, miso, soy yoghurt or soy milk is consumed by the average New Zealander. Intake of soy is therefore low compared with people who frequently eat these soy foods. Documentation of the complete range of New Zealand foods in which soy occurs as an ingredient is not known but soy protein is used in processed meat and soy flour is a minor constituent in bread. Apart from individuals who may consume red clover tablets (as an alternative to hormone replacement therapy), New Zealanders appear not to be exposed to dietary sources of biochanin A or formononetin.

Using consumption information from the model diets used in the New Zealand Total Diet Survey (NZTDS), where available (Cressey *et al*., 2000), or from the 1997 National Nutrition Survey (Russell *et al*.,1999), where available, and both upper and lower concentration data in Tables 2.2.2 and 2.2.3, the maximum and minimum intake for a young male, adult males and adult female New Zealanders can be estimated (Table 2.2.4a and b). For details see Appendix 1.

Table 2.2.4a: Estimated maximum daily dietary intake of genistein (G) and daidzein (D) by age-sex group (mg/day)

Food	Young male		Adult male		Adult female		Vegetarian F	
	G	D	G	D	G	D	G	D
Ham	0.015	0.006	0.025	0.010	0.013	0.005	0.000	0.000
Saveloys	0.038	0.015	0.012	0.005	0.004	0.002	0.000	0.000
Sausages	0.303	0.118	0.208	0.081	0.103	0.040	0.000	0.000
Luncheon	0.009	0.004	0.023	0.009	0.010	0.004	0.000	0.000
Pies, savoury	0.236	0.092	0.236	0.092	0.098	0.038	0.000	0.000
Milk, soy	0.097	0.075	0.279	0.215	0.234	0.181	5.808	4.477
Milk, cow	0.000	0.120	0.000	0.078	0.000	0.057	0.000	0.053
Bread	1.191	0.847	1.242	0.883	0.882	0.628	0.882	0.628
Beer	0.562	0.197	0.637	0.223	0.063	0.022	0.063	0.022
Total	**2.5**	**1.5**	**2.7**	**1.6**	**1.4**	**1.0**	**6.7**	**5.2**

Table 2.2.4b Estimated lower daily dietary intake of genistein (G) and daidzein (D) by age-sex group (mg/day)

Food	Young male		Adult male		Adult female		Vegetarian F	
	G	D	G	D	G	D	G	D
Ham	0.015	0.006	0.025	0.010	0.013	0.005	0.000	0.000
Saveloys	0.038	0.004	0.012	0.001	0.004	0.000	0.000	0.000
Sausages	0.303	0.022	0.208	0.015	0.103	0.008	0.000	0.000
Luncheon	0.009	0.003	0.023	0.007	0.010	0.003	0.000	0.000
Pies, savoury	0.236	0.031	0.236	0.031	0.098	0.013	0.000	0.000
Milk, soy	0.097	0.055	0.279	0.158	0.234	0.132	5.808	3.284
Milk, cow	0.000	0.091	0.000	0.059	0.000	0.043	0.000	0.040

Bread	1.191	0.761	1.242	0.794	0.882	0.564	0.882	0.564
Beer	0.562	0.006	0.637	0.007	0.063	0.001	0.063	0.001
Total	**1.4**	**1.0**	**1.5**	**1.1**	**1.0**	**0.8**	**2.9**	**3.9**

The major contributor of exposure to genistein and daidzein, for the omnivorous diets is bread and for the vegetarian diet is soy milk.

2.2.4 Actual versus theoretical serum levels

Uehar *et al*., (2000) determined mean serum levels of 406.8 nmol/L (110µg/L) of genistein and 118.4 nmol/l (32 µg/l) of daidzein for 111 healthy Japanese women. From dietary records, the mean daily intake for these women was 86.5 µmol/day (23400 µg/day) of genistein and 57.4 µmol/day (15500 µg/day) of daidzein. Assuming serum volume of 2.5l for a woman (Lentner, 1984) and 100% absorption, the theoretical serum level would be dietary intake/serum volume, i.e.:

Genistein: 23400/2.5 =9350 µg/l

Daidzein: 15500/2.5 = 6200 µg/l

The ratio of measured (or actual) :theoretical (A:T) serum levels is:

Genistein: 110/9350 ≈0.01

Daidzein: 32/6200 ≈ 0.005

In another study, mean serum concentrations of 307.5 nmol/l genistein and 111.7 nmol/l daidzein were measured for mean intakes of 111.6 µmol/day genistein and 64.6 µmol/day of daidzein (Arai *et al*., 2000a) with resulting A:T ratios of 0.007 for genistein and 0.004 for daidzein. Wiseman *et al.,* (2004) found mean plasma levels of 187µg/l for genistein and 94µg/l for 13 subjects each consuming 56 mg/day genistein and 43 mg/day daidzein. The sex of these subjects was not specified but for an average plasma volume of 2.9l, these values equate to A:T ratios of 0.01 for genistein and 0.006 for daidzein, in good agreement with the former estimates.

It is apparent from these studies that only a small proportion of ingested isoflavone is actually found in the serum, consistent with efficient 1st pass metabolism in the liver to glucuronides or sulphates that are excreted in the urine (COT, 2003). A:T serum ratios of 0.01 for genistein and 0.005 for daidzein will be used in this assessment of serum

estrogenicity to allow for factors that affect serum levels, such as absorption and metabolism.

2.2.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume, and the difference in molecular weight compared with estradiol, the derived serum estrogenicity of genistein and daidzein for the four New Zealand population subgroups is shown in Table 2.2.5.

Table 2.2.5 Derived maximum and minimum serum XEQ of genistein and daidzein for young males, adult males, females and vegetarian females (µg/l).

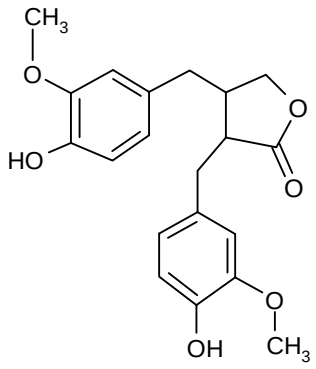
	XEQ (genistein)		XEQ (daidzein)	
Age-sex group	**Maximum (µg/l)**	**Minimum (µg/l)**	**Maximum (µg/l)**	**Minimum (µg/l)**
Young male	2.5×10^{-3}	1.4×10^{-3}	2.1×10^{-4}	2.7×10^{-4}
Adult male	2.4×10^{-3}	1.4×10^{-3}	2.6×10^{-4}	1.7×10^{-4}
Adult female	1.7×10^{-3}	1.2×10^{-3}	2.1×10^{-4}	1.7×10^{-4}
Vegetarian F	8.2×10^{-3}	3.5×10^{-3}	11.1×10^{-4}	8.3×10^{-4}

2.2.6 Uncertainty

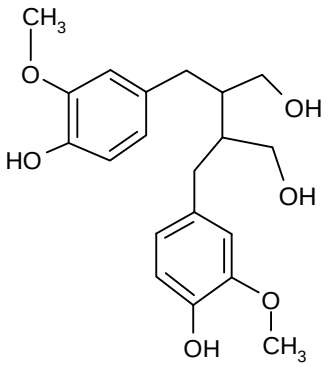
The following qualitative uncertainty assignments are associated with this assessment: food concentration =*, food consumption = *, estrogenic activity = *, A:T = *, Overall uncertainty = *. See Table 2.1.2 for a description of uncertainty assignments.

2.3 Lignans

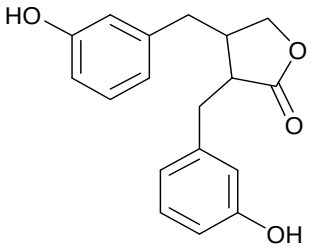
The diphenolic mammalian lignans enterolactone and enterodiol are formed from the plant precursors matairesinol and secoisolariciresinol by gut microflora (Adlercreutz and Mazur, 1997).



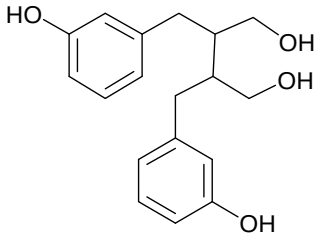
Matairesinol



Secoisolariciresinol



Enterolactone



Enterodiol

Figure 2.2: Chemical structures of the lignans matairesinol and secoisolariciresinol and the metabolites enterolactone and enterodiol

2.3.1 Estrogenic activity

Enterolactone and enterodiol have shown weak estrogenic activity. Enterolactone was approximately one million times less estrogenic than 17β-estradiol (EQ =1 x 10^{-6}) at inducing cell proliferation and enterodiol was approximately 10% as potent as enterolactone (EQ = 10^{-7}) in MCF-7 breast cancer cells (Welshons *et al.*, 1987). These values will be used for the assessment of estrogenicity.

2.3.2 Occurrence in foods

Levels of the plant lignans, matairesinol (an immediate precursor of enterolactone) and secoisolariciresinol (an immediate precursor of enterodiol), have been reported in some pulses, grains, fruits, vegetables and other foods (Adlercreutz and Mazur, 1997, Liggins *et al*., 2000). However balance studies in humans following the consumption of rye bread, have indicated that most of the mammalian lignan precursors remain unidentified (Adlercreutz and Mazur, 1997).

Thus levels of enterolactone and enterodiol produced from food precursors was considered a more realistic measure of dietary exposure to lignans than estimates based on individual plant lignans. The concentrations of enterolactone and enterodiol produced from lignan precursors in a range of 68 plant foodstuffs have been determined by simulated colonic fermentation (Table 2.3.1) (Thompson, 1991). This work showed that by far the richest source was linseed meal from flax (*Linum usitatissimum*), which is currently marketed in various forms as dietary supplements.

Table 2.3.1 Levels of enterolactone and enterodiol produced from simulated colonic fermentation of various foods (Thompson, 1991).

Food	Enterolactone (mg/kg)	Enterodiol (mg/kg)
Apples	0.34	0.01
Asparagus	1.36	2.38
Bananas	0.55	0.14
Bean, broad	0.452	0.308
Bean, kidney	1.155	0.812
Bean, navy	1.232	0.378
Beans, refried or pinto	0.539	0.165
Beef stew	0.264	0.203
Beets	1.09	0.26
Bok choy	0.44	0.14
Bread or rolls, dark	0.648	0.23
Broccoli	1.61	0.65
Cabbage, red	0.3	0.34
Cantaloupe	0.21	0.16
Carrots	2.84	0.62
Cauliflower, brussels sprouts	0.68	0.77
Celery	0.17	0.14
Rolled oats	0.351	0.125
Coleslaw	0.249	0.282
Cornbread	0.617	0.096
Cucumber	0.18	0.11

Food	Enterolactone (mg/kg)	Enterodiol (mg/kg)
Flaxseed (*Linum usitatissimum*), flaxseed bread	31.91	110.32
Garlic	0.81	3.26
Green beans	0.4	0.56
Green pepper	1.62	0.33
Mushrooms	0.43	0.13
Onions	0.11	1.01
Oranges	0.27	0.12
Peanuts, peanut butter	1.05	0.56
Pears	1.12	0.69
Pizza	0.038	0.035
Plums	0.47	0.98
Potatoes, fried	0.33	0.5
Potato, other	0.33	0.5
Potato, sweet (*Ipomoca batata*)	2.4	0.55
Salad, lettuce	0.425	0.55
Snowpeas (*Pisum sativum var, macrocarpon*)	0.6	0.62
Soup, vegetable	0.264	0.203
Soybeans	6.9	1.7
Spaghetti and pasta with tomato sauce	0.039	0.036
Squash (*Cucurbita* spp.)	2.71	1.1
Strawberries	0.41	0.38
Tomatoes	0.11	0.1

2.3.3 Exposure assessment

Dietary intakes for four adult sub-populations were estimated by combining consumption of foods containing lignan precursors from the simulated diets with the levels of enterolactone and enterodiol produced from those foods as detailed in Table 2.3.1. The resulting intake estimates are shown in Table 2.3.2. For details see Appendix 1.

Table 2.3.2 Estimated dietary intakes for the mammalian lignans, enterolactone and enterodiol for population subgroups

Age-sex group	Enterolactone intake (mg/day)	Enterodiol intake (mg/day)
Young male	0.26	0.16
Adult male	0.28	0.15
Adult female	0.19	0.10
Vegetarian female	0.45	0.23

2.3.4 Actual versus theoretical serum levels

Serum concentrations of enterolactone have been reported in a number of studies and are summarized in Table 2.3.3.

Table 2.3.3 Serum concentrations of enterolactone (nmol/l)

Country	gender	Number of subjects	Enterolactone concentration nmol/l	Enterodiol concentration nmol/l
Finland	men	2380	13.8 [1]	na
	women		16.0[1]	na
USA	women	60	20.2[2]	1.52
USA	women	115	13.3 [3]	na
	men	78	11.0 [3]	na
Finland	men	488	15.5 [4]	na
Sweden	men	342	13.8 [4]	na
Norway	men	1720	6.6[4]	na

na = not available, 1 = Kilkkinen *et al.*, 2001 2 = Zeleniuch-Jacquotte *et al* ., 1998, 3 = Horner *et al*., 2002 4 = Stattin *et al*., 2002.

Although serum concentrations of enterolactone have been correlated with the intake of vegetables, fruits, berries, whole grains, caffeine containing drinks and alcohol (Kilkennan *et al*., 2001,2003 Horner *et al*., 2002) neither of these studies report intake of enterolactone precursors with serum concentrations. However, in one Finnish study, 75 non-smoking men were given rye bread containing variable amounts of lignan precursors (Vanharanta *et al*., 2002). Intake and serum levels are shown in Table 2.3.4 with derived theoretical serum concentrations assuming 100% absorption and no metabolism.

Table 2.3.4 Dietary intake, serum concentration (Vanharanta *et al*., 2002) and derived theoretical serum concentration of enterolactone

Intake of lignan precursors (nmol/day)	Actual serum (A) concentration (nmol/l)	Theoretical (T) serum concentration (nmol/l)	A:T
998	1.9	302	0.006
12331	25.3	34040	0.0007
20773	27.1	6295	0.004

For this assessment of serum estrogenicity, a worst case A:T ratio of 0.005 will be assumed for both enterolactone and enterodiol.

2.3.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume, the derived serum estrogenicity of enterolactone and enterodiol for the four New Zealand population subgroups is shown in Table 2.3.5.:

Table 2.3.5 Derived serum XEQ of enterolactone and enterodiol for adult males, females and young males (µg/l).

Age-sex group	Enterolactone intake (mg/day)	XEQ (µg/l)	Enterodiol intake (mg/day)	XEQ (µg/l)
Young male	0.26	4.1×10^{-7}	0.16	2.7×10^{-8}
Adult male	0.28	4.2×10^{-7}	0.15	2.3×10^{-8}
Adult female	0.19	3.5×10^{-7}	0.10	2.0×10^{-8}
Vegetarian female	0.45	8.2×10^{-7}	0.23	4.1×10^{-8}

2.3.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration =**, food consumption = *, estrogenic activity = **, A:T = *, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.4 Coumestans

Coumestans are phytoestrogens structurally similar and biosynthetically related to isoflavones. A large number of coumestans have been isolated from plants, but relatively few have been shown to have estrogenic activity. The only coumestan for which estrogenic and dietary information is available is coumestrol (Figure 2.3), present in soya beans, alfalfa, clover and other fodder crops.

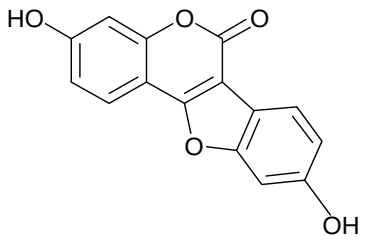


Figure 2.3: Chemical structure of the phytoestrogen coumestrol

Coumestrol is structurally similar to 17β-estradiol with two distal hydroxyl groups attached to a four fused ring structure. There are also close structural similarities to the other phytoestrogens (chalcones, flavones, and flavonols).

2.4.1 Estrogenic activity

The limited data for the estrogenicity of coumestrol relative to estradiol has been summarised in Table 2.4.1. The estrogenic potency of coumestrol is extremely variable**.** The greatest reported estrogenicity relative to estradiol, using a cell proliferation assay is EQ= 0.0003 (300 x 10^{-6}). This value will be used for the current risk assessment.

Table 2.4.1 Estrogenic potencies of coumestrol relative to estradiol

	***In vitro* assays**			***In vivo* assays**
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1	1	**1**	1
Coumestrol	0.1[1] 0.021[2]	0.002[3]	**0.0003[4]** **0.00001[5]**	0.00035[1] 0.000001[6]

1=IEH, 2000 2=Hopert *et al.*, 1998 3=Markiewicz *et al.*, 1993 4=Mayr *et al.*, 1992
5=Soto *et al.*,1995 6= Milligan *et al.*, 1998

2.4.2 Occurrence in foods

Coumestrol is infrequently found in foods but it has been reported in alfalfa sprouts, kala chana seeds, split peas, lima beans, pinto beans, and soyabean sprouts. Analysis of coumestans in New Zealand foods has been limited to six herbal supplements with the concentration of coumestrol ranging from <7 to 1285 mg/kg (Taylor and Burlingame, 1998). The limited data on occurrence of coumestrol in foods and dietary supplements is shown in Table 2.4.2.

Table 2.4.2: Average concentration of coumestrol in foods.

Food type	Coumestrol content (mg/kg)
Alfalfa sprouts[1] (M*edicago sativa)*	46.8
Alfalfa tablets[2]	48.9-1285
Alfalfa and kelp[2]	847
kala chana seeds[1], (chickpea)(*Cicer arietinum)*	61.3
split peas[1] (*Pisum sativum*)	81.1
lima beans[1] (*Phaseolus limensis)*	ND-14.8
soyabean sprouts[1]	4.5-12.1

1=IEH, 2000 2=Taylor and Burlingame, 1998

2.4.3 Exposure assessment

The foods in which coumestrol has been shown to occur are generally foods which are not frequently consumed by the wider New Zealand population. Consumption of kala chana seeds, lima beans, pinto beans and soyabean sprouts was not reported by any of 4636 subjects who completed 24 hour dietary recall questionnaire as part of the National Nutrition Survey (NNS) (Russell *et al*., 1999). Certain assumptions are required in order to make an estimate of exposure of New Zealanders to coumestrol. For the foods not reported in the NNS an assumption was made that one person in 5000 would consume 50 g of these foods on a daily basis. This equates to a daily level of consumption for the average New Zealander of 0.01 g/day. Both maximum and minimum intakes were calculated using the concentration range data in Table 2.4.2 but since consumption of these foods is very low, the maximum and minimum intakes were equivalent. The estimated coumestrol exposures were 0.033, 0.019, 0.027 and 0.027 mg/day for young males, adult males, adult females and vegetarian females respectively. Consumption estimates for alfalfa sprouts and split peas were based on actual NNS data. Since NNS

data does not distinguish vegetarian females, exposure of this group to coumestrol was assumed to be the same as for adult females. For details see Appendix 1.

Those individuals who choose to consume alfalfa tablets are likely to be exposed to highly elevated amounts of coumestrol compared with the majority of the population for whom any risk is most unlikely.

2.4.4 Actual versus theoretical serum levels

There are no reports of human serum levels of coumestrol.

2.4.5 Serum estrogenicity XEQ

Given the structural similarity between coumestrol and the isoflavones, especially daidzein, the worst case (higher) A:T ratio of these two isoflavones i.e. 0.01 will be used in the current assessment. Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume, the derived serum estrogenicity of coumestrol is 1.7, 3.3 and 3.5 x 10^{-5} for adult males, females and young men respectively.

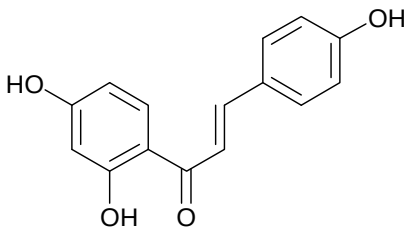
2.4.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration =**, food consumption = **, estrogenic activity = **, A:T = ***, Overall uncertainty = ***. See Table 2.1.2 for a description of uncertainty assignments.

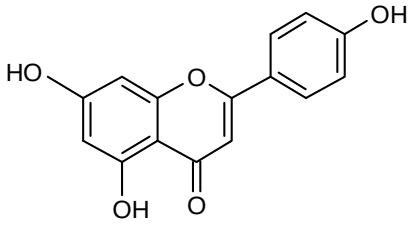
2.5 Flavonoids

Flavonoids are a broad group of compounds including chalcones, flavones, flavonols and flavanones, which includes many of the most common plant pigments. Isoflavones are a subgroup of the flavonoids, but have been covered separately (Chapter 2.2). Of the wide range of compounds in this category, the three most commonly reported in food are the flavonols quercetin, kaempferol and myricetin. Important dietary sources of flavonoids are vegetables, fruits and beverages.

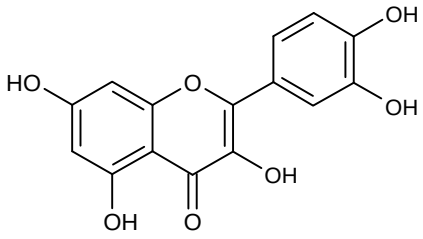
Varying numbers and positions of hydroxyl groups leads to a wide range of chemically related compounds. Hydroxylation in the 4' position is a common feature of the estrogenic flavonoids. The structure of example flavanonoids are shown below.



Isoliquiritegenin (a chalcone)



Apigenin (a flavone)



Quercetin (a flavonol)

Figure 2.4: Chemical structures of isoliquiritigenin, apigenin and quercetin, representing examples of flavonoids found in food.

2.5.1 Estrogenic activity

A summary of literature reports of estrogenicity of seven flavonoids relative to 17β-estradiol, in receptor binding, gene expression and cell proliferation assays, are shown in Table 2.5.1.

Table 2.5.1 Relative estrogenic potencies of flavonoids

		***In vitro* assays**	
	Receptor binding	**Gene expression**	**Cell proliferation**
17β-estradiol	1	1	**1**
Quercetin	0.00001[1]	0.03[2]	**<0.000001[4]**
	0.0004[2]	<0.0005[1]	
Kaempferol	0.00012[1]	0.005[1]	**0.00001[4]**
	0.03[2]	0.5[2]	**0.00007[5]**
		0.004[3]	
		0.000001[5]	
Apigenin	0.06[2]	0.5[2]	**0.00005[4]**
		0.03[3]	**0.00015[5]**
		0.000002[5]	
Luteolin		0.0009[3]	**0.00001[4]**
			0.00006[5]
Isoliquiritigenin		0.005[3]	
Naringenin	0.0011[2]	0.5[2]	**0.0001[4]**
		0.003[3]	**0.00008[5]**
		0.00008[5]	
Phloretin	0.007[2]	0.5[2]	**0.000025[5]**
		0.002[3]	
		0.0000009[5]	

1=Zava *et al.*, 1997 2=Kuiper *et al.*, 1998. The values given are the highest reported where estrogenicity varied between the α and β estrogen receptor subtypes.
3=Miksicek, 1995 4=Le Bail *et al.*,1998 5=Breinholt and Larsen,1998

Estrogenic potency of flavonoids will be based on the cell proliferation assay results reported by Le Bail *et al.*, (1998) and Breinholt and Larsen (1998). Results from the two studies are generally in good agreement. Where they differed the higher potency value will be adopted, in line with the conservative approach taken in this study. Thus apigenin = 150 x 10^{-6}, luteolin = 60 x 10^{-6}, kaempferol = 70 x 10^{-6}, naringenin = 100 x 10^{-6} and phloretin = 25 x 10^{-6}. Where no results are available (isoliquiritigenin), a relative estrogenic potency equal to the most estrogenic flavonoid (apeginin, EQ= 0.00015) (150 x 10^{-6}) will be assigned, therefore this represents the worst case scenario from a risk perspective. An EQ value of 0.000001 (1 x 10^{-6}) is assigned for quercetin.

2.5.2 Occurrence in foods

There are no data available on the level of chalcones, flavones, flavanols or flavanones in New Zealand foods. However, extensive work has been carried out overseas on the analysis of flavonoids in foods by Hertog and co-workers (Hertog *et al.*, 1992; 1993). In these studies the three major flavonols, quercetin, kaempferol and myricetin, and two major flavones, luteolin and apigenin were selected for analysis. The reason for selection of these constituents was not because of estrogenic potential, but rather, because of the anticarcinogenic properties of these compounds. Losses of 50 to 100% were observed for processed compared with unprocessed food (ie there were higher concentrations in raw than cooked food). Two compilations of literature values for the concentration of five flavanols and flavones in human foods have been published (IEH, 2000; Pillow *et al.*, 1999). A summary of available relevant analytical data is shown (Table 2.5.2).

Table 2.5.2: Literature values for the concentration of flavonoids in human foods (mg/kg).

	Quercetin	Kaempferol	Myricetin	Apigenin	Luteolin
Apples	36	<2	<1	<2	<1
Apple juice[1]	2.8	<1	<0.5	<1	<0.5
Apricot	25	<2	<1	<1	<1
Broad beans	20	<2	26	<2	<1
Broccoli	30	72	<1	<1	<1
Celery	<1	<2	<1	108	22
Endive	<1	46	<1	<2	<1
Grape	12-15	<2	4.5	<1	<1
Green beans	29-39	<12	<1	<1	<1
Green pepper	18	<1	<1	<1	<1
Kale	110	211	<1	<1	<1
Leek	<1	30	<1	<1	<1
Lettuce	14	<2	<1	<1	<1
Onion	347	<2	<1	<2	<1
Orange juice	3.4-5.7	<1	<1	<1	<1
Pear	6.4	<2	<1	<1	<1
Plum	9	<2	<1	<1	<1
Red cabbage	4.6	<2	<1	<1	<1
Red currant	13	<2	<1	<1	<1
Red pepper	<1	<1	<1	<1	11
Red wine	11	<1	9	<1	<0.5
Strawberry	8.6	12	<1	<1	<1
Sweet cherry	15	<2	<1	<1	<1
Tea[1]	20	14	2.5	<1	<0.5
Tomato	8.0	<2	<1	<1	<1
Tomato juice[1]	13	<1	<0.5	<1	<1

1=Reported in mg/l ≈mg/kg

Isoliquiritigenin occurs in liquorice and soybeans but concentrations were not available. This flavonoid occurs mainly in the plant root and, consequently, is unlikely to enter the diet in any food except liquorice. Weinberg *et al*., (1993) reported isoliquiritigenin at levels of 9600 mg/kg in liquorice-root extract powder.

Naringenin is the aglycone of naringin and is a constituent of grapefruit, tangerines and bitter oranges (Widmer, 2000). The concentration of total naringin in grapefruit is generally in the range 290-480 mg/kg (Gray and Olson, 1981; Hsu *et al*., 1998; Widmer, 2000), while grapefruit juice has been reported to have slightly lower levels (115-384 mg/kg; Ho *et al*., 2000).

Phloretin occurs solely in apples and is used as a marker for contamination of fruit juices and jams by apple products (Fernández de Simón *et al*., 1992; Andrade *et al*., 1998). Phloretin is principally found in apples in the form of the xyloglucoside and the glucoside (phloridzin) (Burda *et al*., 1990; Spanos *et al*., 1990; Andrade *et al*., 1998) and occur at levels 6-8 times higher in the skin than the flesh of the apple (Burda *et al*., 1990). Levels of total phloretin in apples are typically 20-40 mg/kg (Burda *et al*., 1990; Andrade *et al*., 1998) and in apple juice typically 10-75 mg/kg (Burda *et al*., 1990; Spanos *et al*., 1990; Spanos and Wrolstad, 1992; Andrade *et al*., 1998).

2.5.3 Exposure assessment

Both maximum and minimum flavonoid exposures were calculated from the concentration data presented in Table 2.5.2 and the range data for phloretin concentrations in apples and apple juice. For the purpose of calculating a dietary exposure to isoliquiritigenin it is assumed that the level in confectionery liquorice is the same as that found in liquorice-root extract powder. Estimates of dietary exposure to seven estrogenic flavonoids, largely based on consumption figures from the model diets of the NZTDS, are given in Table 2.5.2. Consumption of liquorice was based on data from the 1997 National Nutrition Survey (Russell *et al*., 1999) and was assumed to be the same for all population subgroups. For details see Appendix 1.

Table 2.5.3: Estimated dietary exposure to six flavonoids for four population subgroups (mg/day)

	Young male	Adult male	Adult female	Vegetarian female
Quercetin	6.7-6.9	8.6-8.8	9.6-9.8	9.4-9.5
Kaempferol	1.8	3.5	5.0	3.0
Luteolin	0.10	0.10	0.14	0.10
Apigenin	0.31	0.31	0.54	0.31
Naringenin	2.9	2.9	1.8	1.8
Phloretin	1.6-6.8	1.1-3.7	0.9-3.2	1.0-4.7
Isoliquiritigenin	2.3	2.3	2.3	2.3
Total	**15.7-21.1**	**18.8-21.4**	**20.3-22.78**	**17.9-21.7**

2.5.4 Actual versus theoretical serum levels

In a Dutch study, 8 male and 7 female subjects were given strong black tea, providing 49 mg quercetin and 27 mg kaempferol, or fried onions, providing 13 mg quercetin (de Vries *et al*, 1998). The mean serum concentration of quercetin, was 29µg/l from the consumption of tea, and 22µg/l from the onions (compared with a baseline level of 7 µg/l). The mean serum level of kaempferol was 15 µg/l. Intake and serum levels are shown in Table 2.5.3 with theoretical serum concentrations assuming 100% absorption, no metabolism and a serum volume of 2.7l (the average of 3.3 and 2.5l for males and females respectively).

Thirty seven female subjects fed a high vegetable diet contributing 29 mg/day of naringenin were found to have a mean serum concentration of 29.4 µg/l above a baseline level (Erlund *et al.,* 2002). The theoretical serum level from 29 mg/day is 11600 (29 x 1000/2.5) µg/l, therefore A:T = 0.003. Phloretin was not detected (<9.9 µg/l) in serum samples from six subjects (4 male and 2 female) who had consumed 4.8 mg of phloretin precursors from the consumption of apple cider (DuPont *et al*., 2002). Assuming a serum level at the limit of detection, and a mean serum volume of 3.0l, for 4 males and 2 females, a maximum A:T ratio of 0.006 is derived. No data on serum levels of apigenin, isoliquiritigenin or luteolin is available.

Table 2.5.4 Dietary intake versus serum concentration of flavonoids

Flavanoid	Intake of flavanoid (mg/day)	Actual serum (A) concentration (µg/l)	Theoretical (T) serum concentration (µg/l)	A:T
quercetin	49[1]	22	18148	0.001
	13[1]	15	4815	**0.003**
kaempferol	27[1]	15	10000	**0.0015**
naringenin	29[2]	29.4	11600	**0.0025**
phloretin	4.8[3]	<9.9	1584	**0.006**

1=de Vries *et al*., 1998 2=Erlund *et al*., 2002. 3= DuPont *et al*., 2002.

The A:T ratios of all the flavonoids considered, including genistein (0.01) and daidzein (0.005), are within one order of magnitude. For the assessment of serum estrogenicity the assumed A:T ratios have been highlighted in bold font in Table 2.5.4. Where A:T ratios cannot be derived because there is insufficient information (apigenin, luteolin and isoliquiritigenin), an A:T ratio of 0.01 is assumed , as this reflects the worst case scenario for serum estrogenicity.

2.5.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of the various flavonoids for the three New Zealand populations are shown in Table 2.5.5.

Table 2.5.5 Derived serum XEQ of flavonoids for a young male, adult males, females and a vegetarian female (µg/l).

Flavanoid	Young male	Adult male	Adult female	Vegetarian female
Quercetin	$6.2\text{-}6.4 \times 10^{-6}$	$7.0\text{-}7.2 \times 10^{-6}$	$1.0\text{-}1.1 \times 10^{-5}$	1.0×10^{-5}
Kaempferol	6.2×10^{-5}	1.1×10^{-4}	2.0×10^{-4}	1.2×10^{-4}
Luteolin	2.0×10^{-5}	1.7×10^{-4}	3.2×10^{-5}	2.2×10^{-5}
Apigenin	1.6×10^{-4}	1.4×10^{-4}	3.3×10^{-4}	1.9×10^{-4}
Naringenin	3.0×10^{-4}	2.6×10^{-4}	2.2×10^{-4}	2.2×10^{-4}
Phloretin	$0.8\text{-}3.5 \times 10^{-4}$	$0.5\text{-}1.7 \times 10^{-4}$	$0.5\text{-}1.9 \times 10^{-4}$	$0.6\text{-}2.8 \times 10^{-4}$
Isoliquiritigenin	1.3×10^{-3}	1.1×10^{-3}	1.5×10^{-3}	1.5×10^{-3}

2.5.6 Uncertainty

The qualitative uncertainty assignments associated with each of the flavonoids of this assessment are shown in Table 2.5.6.

Table 2.5.6: Qualitative uncertainties associated with the exposure estimate for flavonoids.

Flavanoid	Food concentration	Food consumption	Estrogenic activity	A:T ratio	Overall uncertainty
Quercetin	**	*	**	*	**
Kaempferol	**	*	*	*	**
Luteolin	**	*	**	***	***
Apigenin	**	*	*	***	***
Naringenin	**	*	**	*	**
Phloretin	**	*	*	*	**
Isoliquiritigenin	**	*	***	***	***

A definition of uncertainty assignments is given in Table 2.1.2.

2.6 Resorcyclic lactones

These compounds are secondary fungal metabolites, produced by several *Fusarium* species that proliferate in poorly stored grains, oilseeds and hay. *Fusarium* infection tends to develop during prolonged cool, wet, growing and harvest seasons (FAO, 1999). The most important resorcyclic lactone, zearalenone, occurs worldwide with the highest incidence rates occurring in North America, and central and northern Europe. But the occurrence of zearalenone has also been reported in Egypt, South Africa, Italy South America and New Zealand (FAO, 1999). In mammals, zearalenone is metabolized to the stereoisomers, α and β zearalenol (Figure 2.5). Some structural similarities to 17β-estradiol are apparent, with two distally located oxygen-containing groups.

zearalenone *(α, β)-zearalenol*

Figure 2.5: Chemical structure of zearalenone and its metabolies α and β-zearalenol

2.6.1 Estrogenic activity

Zearalenone and zearalenol are two of the most potent xenoestrogens. A summary of available estrogenic potency data is shown in Table 2.6.1. There is fairly good agreement around various estimates of the estrogenic potency of zearalenone and the commonly derived estimate of 1% of the activity of 17β-estradiol (EQ = 0.01) will be used for the current study. Although there is some evidence to suggest that the relative activity of zearalenol may be greater, the current risk assessment will deal only with zearalenone as this is the form of the lactone for which most information is available and the form in which it is mainly ingested.

Table 2.6.1: Summary of estrogenic potencies (EQ) determined for zearalenone and zearalenol

	In vitro assays			*In vivo* assays
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1		**1**	1
Zearalenone	0.01[1]		**0.01**[3]	0.006-0.013[5]
	0.033-0.034[2]		**0.01**[4]	
Zearalenol			**0.01**[3]	
			0.56[4]	

1=Kiang *et al.*, 1978 2= IEH, 2000 3= Soto *et al.*, 1995
4= Welshons *et al.*, 1990 5= Hobson *et al.*, 1977

A relative estrogenic potency of EQ = 0.01 (10,000 x 10^{-6}) will be used in the current risk assessment.

2.6.2 Occurrence in foods

In New Zealand, the climate is generally favourable for the growth of *Fusarium* species and zearalenone (and trichothecene mycotoxins) were found in a preliminary investigation of wheat (Agnew *et al.*, 1986) and in a small study of maize (Hussein *et al.*, 1989). In a large survey of the occurrence of *Fusarium* mycotoxins in cereals grown in New Zealand in 1986-1989, Lauren *et al.,* (1991) reported the common finding of zearalenone in maize samples (75%) but at relatively low levels, none greater than 500 µg/kg. Wheat, barley and oats from 6 regions in New Zealand showed contamination rates of 32%, 18% and 34% respectively with maximum concentrations of 460, 170 and 90 µg/kg. Samples of commercially grown hybrids of maize taken at harvest from several North Island sites from 1992 to 1994 were analysed for *Fusarium* mycotoxins including zearalenone (Lauren *et al.*, 1996). Results are summarised in Table 2.6.2. Two factors contributing to higher contamination levels were the late harvesting of crops and the use of hybrids that were more susceptible to accumulation of zearalenone.

Table 2.6.2: Distribution of zearalenone contamination of New Zealand maize

Year	**No. samples**	**% of samples with zearalenone (mg/kg)**				
		<0.4	**0.4 – 1.0**	**1.0 – 2.0**	**>2.0**	**Max. level**
1992	178	67	17	8	7	10.5
1993	162	81	14	3	2	2.7
1994	176	93	3	2	2	6.1

Internationally, there have been many reports of contamination of foods (Price and Fenwick, 1985 and Pohland and Wood,1987).

A preliminary survey of maize-based foods, mainly cornflakes, was conducted by HortResearch (The Horticulture and Food Research Institute of New Zealand Ltd.) for samples collected in 1993. The range in 7 samples (5 positives) was from 0.16 to 0.30 mg/kg with a mean concentration of 0.22 mg/kg. An extensive survey in 1995 examined 124 foods and found a much lower incidence of contamination (7.2%) (Lauren and Veitch, 1996). Results are presented in Table 2.6.3.

Table 2.6.3 Zearalenone concentrations (mg/kg) of maize-based foods in New Zealand (Lauren and Veitch, 1996)

Food category	No. samples	No. positives	Range (mg/kg)
Breakfast Foods	20	5	0.05 – 0.12
Extruded snack foods	20	1	0.14
Flours; grits	17	2	0.12 – 0.30
Breads	16	2	0.06 – 0.14
Masa flour products	24	0	< 0.05
Corn (maize) oils & snack bars	21	0	< 0.05
Corn syrups & liquid brewing sugar	6	0	< 0.05

2.6.3 Exposure assessment

In New Zealand exposure to zearalenone is likely to be principally through consumption of corn-based foods with breakfast cereals, mixed grain breads and extruded corn-based snack foods likely to be the major contributors

Table 2.6.4 combines food consumption information from simulated diets formulated for the 1997/98 NZTDS with average zearalenone concentrations for mixed grain breads, corn-based breakfast cereals and corn-based snack foods from the study of Lauren and Veitch (1996). Average zearalenone concentrations were calculated by assuming that some level of zearalenone was present in all samples and assuming that the level in samples reported as 'not detected' was equal to half of the limit of detection.

Table 2.6.4: Estimated dietary exposure to zearalenone for four population age-sex groups

Age-sex group	Food	Consumption (g/day)	Zearalenone concentration (mg/kg)	Exposure (mg/day)
Adult male	Mixed grain bread	25	0.034	0.00085
	Breakfast cereal	2.9	0.04	0.00012
	Total			**0.00097**
Adult female	Mixed grain bread	17	0.034	0.00058
	Breakfast cereal	4.3	0.04	0.00017
	Total			**0.00075**
Young male	Mixed grain bread	29	0.034	0.00099
	Breakfast cereal	4.4	0.04	0.00018
	Total			**0.00117**
Vegetarian female	Mixed grain bread	62	0.034	0.00211
	Breakfast cereal	4.3	0.04	0.00017
	Total			**0.00228**

2.6.4 Actual versus theoretical serum levels

There are very few reports of human serum levels of zearalenone. In a study undertaken in South Africa, the mean serum level of zearalenone for 132 female subjects was 0.36 μg/ml (360 μg/l) (Pillay *et al*., 2002). This level included glucuronides and is therefore an overestimate of free (estrogenic zearalenone) but does not include the estrogenic derivatives α- and β-zearalenol that were also detected. The study made no assessment of dietary intake of zearalenone. An intake of 0.00075 mg/day (as estimated for a New Zealand female) would equate to a theoretical serum level of 0.3μg/l. This is 1000 times lower than what was measured in the South African study. The intake in South Africa might be in the order of 10-100 times higher because of a higher concentration of zearalenone in South African corn (Pillay *et al*., 2002, Price and Fenwick, 1985, Pohland and Wood,1987) and a greater consumption of corn in South Africa compared with New Zealand where it is less of a staple food. However, this does not completely account for the higher serum levels measured than might be expected. It may be that zearalenone accumulates in the body. In the absence of better data, an A:T ratio of 1 is assumed for the purposes of this assessment of estrogenicity.

2.6.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume, the derived serum estrogenicity of zearalenone for the four New Zealand population subgroups is shown in Table 2.6.5.

Table 2.6.5: Derived serum XEQ of zearalenone for young males, adult males, females and vegetarian females (µg/l).

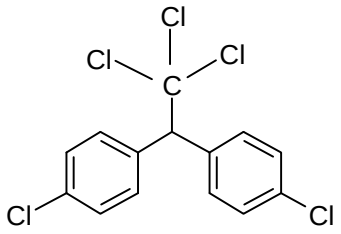
Age-sex group	Zearalenone intake (mg/day)	XEQ (µg/l)
Young male	0.00117	3.5×10^{-3}
Adult male	0.00097	2.5×10^{-3}
Adult female	0.00075	2.6×10^{-3}
Vegetarian female	0.00228	7.8×10^{-3}

2.6.6 Uncertainty

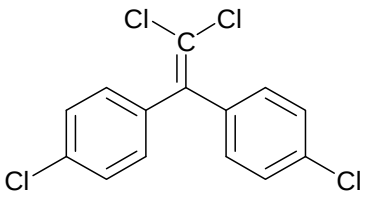
The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = **, A:T = ***, Overall uncertainty = ***. See Table 2.1.2 for a description of uncertainty assignments.

2.7 DDT and metabolites

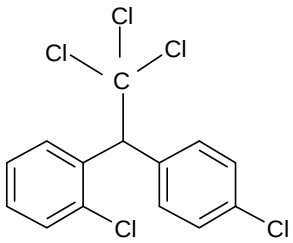
The insecticide, *p,p'*-DDT, was used in New Zealand, primarily for the control of grass grub, from the late 1940s. Use of DDT on farmland in New Zealand has been prohibited since the 1970s when it became apparent that it was accumulating in the environment and the foodchain. Commercial DDT comprised a mixture of isomers, the main component being *p,p'*-DDT, and up to 30% *o,p'* DDT. In the environment and *in vivo,* DDT breaks down, or is metabolised to *p,p'* DDE and *p,p'* DDD (also known as TDE). The structure of *p,p'*-DDT, *o,p'* DDT and metabolites, *p,p'*-DDE and *p,p'*-TDE are shown below.



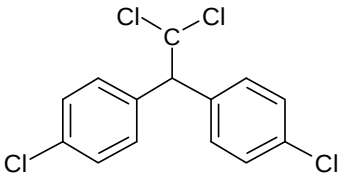
p,p' DDT



p,p' DDE



o,p' DDT



p,p' TDE

Figure 2.6: Chemical structures of *p,p'*-DDT, *o,p'* DDT and metabolites, *p,p'*-DDE and *p,p'*-TDE

2.7.1 Estrogenic activity

There is consistent evidence of the estrogenicity of *o,p'* DDT and some evidence for estrogenicity of *o,p'* TDE, *o,p'* DDE and *p,p'* DDT. There is more limited evidence for the estrogenicity of *p,p'* TDE. There is no evidence of estrogenic effects from the major metabolite found in foods (*p,p'* DDE). A summary of estrogenic potency of DDT and its metabolites, relative to 17β-estradiol, is shown in Table 2.7.1.

Table 2.7.1: Relative estrogenic potencies of DDT and metabolites

	In vitro assays			*In vivo* assays
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1	1	1	1
p,p' DDT	0.001[1]	0.00003[3]	**0.000001[2]**	
*o,p'*DDT	0.001[1]	0.0014-0.003[1]	**0.000001[2]**	0.0001-0.001[4]
	0.000001[2]	0.00011[3]		
o,p' DDE	0.001[1]	0.00004[3]		
p,p' TDE	0.00001[5]			
o,p' TDE	0.001[1]			

1=Chen *et al.,* 1997 2=Soto *et al.,* 1995
3=Coldham *et al.,* 1997 4=IEH, 1995 5=Klotz *et al.,* 1996

A relative potency of EQ=0.000001 (1 x 10^{-6}) will be used to assess estrogenicity from DDT and its metabolites, but excluding the non estrogenic *p,p* DDE.

2.7.2 Occurrence in foods

The parent compound, *p,p'*-DDT, and its metabolites, *p,p'*-DDE and *p,p'*-TDE have been found in decreasing concentrations in the five NZTDSs from 1974/75 to 1997/98 (Cressey *et al.*, 2000). *p,p'*-DDT was found at a mean concentration of 0.003 mg/kg in a composite egg sample and *p,p'*-DDE was found in 22 foods of the 1997/98 NZTDS, primarily in foods of animal origin, as shown in Table 2.7.3. The isomer *o,p'*- DDT and the metabolites *o,p'*- DDE, *p,p'*-TDE and o,*p'*-TDE were not detected in any food samples in the 1997/98 NZTDS (Vannoort *et al.,* 1997, 1998a,b,c).

2.7.3 Exposure assessment

The following assumptions were made to estimate dietary exposure to DDT and its metabolites (excluding the non estrogenic, but more prevalent *p,p'*-DDE). For those food items which were found to contain *p,p'*-DDE in the 1997/98 NZTDS (listed in Table 2.7.3), it is assumed that these foods also contained some level of *o,p'*-DDT, *p,p'*-DDT, *o,p'*-TDE, *p,p'*-TDE and/or *o,p'*-DDE at levels below the limit of detection (0.003 mg/kg for each analyte; Cressey *et al.*, 2000). Since the relative estrogenic potency of *p,p'*-TDE appears to be about 10 times less than the other analytes the risk from this analyte would be less (by a factor of 10 times) and therefore it is not included in the exposure estimate for combined DDT analytes. Assuming the remaining DDT analytes are present at the limit of detection (0.003 mg/kg), an assumed combined concentration of 0.012 mg/kg for

these four DDT analytes will be used to derive exposure estimates. This is a conservative approach and will lead to a considerable overestimation of exposure to estrogenic DDT metabolites. Estimated dietary exposures to estrogenic DDT for adult males, adult females, young males and vegetarian females are 0.0055, 0.0036, 0.0073 and 0.0019 mg/day respectively (Table 2.7.3). This estimate includes the low level (0.003 mg/kg) of *p,p'*-DDT found in eggs (Cressey *et al.*, 2000).

Table 2.7.3: Estimated daily exposure to estrogenic DDT analytes (*o,p'*-DDT, *p,p'*-DDT, *o,p'*-TDE and *o,p'*-DDE) for three sub populations (µg/day).

Food	Adult male		Adult female		Young male		Vegetarian female	
	Eaten g/day	Intake µg/day	Eaten g/day	Intake µg/day	Eaten g/day	Intake µg/day	Eaten g/day	Intake µg/day
Bacon	5.0	0.06	3.6	0.04	4.3	0.05	0.0	0.00
Beef, mince	12.9	0.15	7.9	0.09	13.6	0.16	0.0	0.00
Beef, rump	12.9	0.15	7.9	0.09	12.9	0.15	0.0	0.00
Biscuits, chocolate	17.9	0.21	12.5	0.15	20.0	0.24	12.0	0.14
Butter	15.9	0.19	10.3	0.12	18.9	0.23	32.9	0.39
Cheese	20.0	0.24	18.9	0.23	29.9	0.36	0.0	0.00
Chicken	12.9	0.15	10.0	0.12	12.1	0.15	0.0	0.00
Chicken nuggets	7.1	0.09	4.3	0.05	7.9	0.09	7.7	0.09
Egg	21.8	0.26	13.9	0.17	16.5	0.20	22.7	0.27
Fish in batter	12.9	0.15	4.3	0.05	10.4	0.12	0.0	0.00
Fish, terakihi	8.6	0.10	4.3	0.05	5.0	0.06	0.0	0.00
Hamburger, plain	16.1	0.19	16.1	0.19	19.3	0.23	0.0	0.00
Lamb/mutton, leg	12.9	0.15	9.3	0.11	13.6	0.16	0.0	0.00
Lamb/mutton, shoulder	25.7	0.31	14.3	0.17	26.4	0.32	0.0	0.00
Lamb's liver	2.1		1.8	0.02	2.5	0.03	0.0	0.00
Luncheon sausage	10.0	0.12	7.5	0.09	10.7	0.13	0.0	0.00
Milk, whole	186.4	2.24	116.4	1.40	327.9	2.24	77.9	0.93
Pie, meat	11.4	0.14	8.6	0.10	11.4	0.14	0.0	0.00
Pizza	16.1	0.19	14.3	0.17	17.9	0.21	0.0	0.00
Pork pieces	11.4	0.14	8.6	0.10	14.3	0.17	0.0	0.00
Raisins/Sultanas	1.1	0.01	1.4	0.02	1.8	0.02	3.6	0.04
Sausages, beef	12.9	0.15	6.1	0.07	12.9	0.15	0.0	0.00
Total µg/day		5.45		3.62		7.32		1.88
mg/day		**0.0055**		**0.0036**		**0.0073**		**0.0019**

ND = not detected

2.7.4 Actual versus theoretical serum levels

The level of DDT and its metabolites have been measured in the serum of non occupationally exposed New Zealanders (Buckland *et al.*, 2001). Serum from 1834 subjects was pooled into 60 samples. Serum concentrations of *p,p'* DDT and *o,p'* DDT, and the serum lipid levels from which these values were derived, are shown in Table 2.7.4. The major metabolite *p,p'* DDE was detected, but is not included because it is not estrogenic.

Table 2.7.3: Maximum serum concentrations of estrogenic DDT analytes in non-occupationally exposed New Zealanders (µg/l) (from Buckland *et al.*, 2001).

	15-24 years	25-34 years	35-49 years	50-64 years	65+ years
Female					
o,p' DDT		0.14	0.14	0.16	**0.18**
p,p' DDT		0.20	0.18	0.35	**0.43**
Serum lipid (%w/v)		0.68	0.68	0.79	0.88
Male					
o,p' DDT	**0.15**	0.08	**0.17**	0.09	0.08
p,p' DDT	**0.11**	0.19	**0.28**	0.21	0.17
Serum lipid (%w/v)	0.77	0.79	0.86	0.87	0.83

Combining the maximum serum values for the two DDT analytes, representing upper exposure, and intakes from Table 2.7.3, the following A:T ratios are derived:

Table 2.7.4: Dietary intake versus serum concentration and derived serum concentration of estrogenic DDT analytes.

Sub population	Intake of DDT analytes (mg/day)	Actual serum (A) concentration (µg/l)	Theoretical (T) serum concentration (µg/l)	A:T
Adult male	0.0055	0.45	1.7	0.26
Adult female	0.0036	0.61	1.4	**0.44**
Young male	0.0073	0.26	2.5	0.10

2.7.5 Serum estrogenicity XEQ

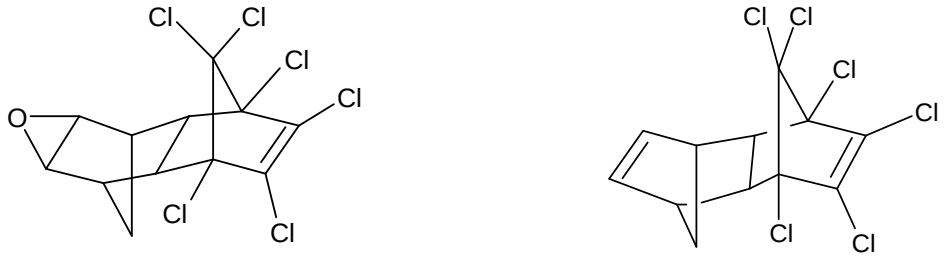
Combining exposure with a conservative A:T ratio of 0.44, relative estrogenic potency (1×10^{-6}) and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of DDT analytes for the three New Zealand populations is 5.6, 4.9, 8.5 and 2.6×10^{-7} µg/l for adult males, females, young males and vegetarian females respectively.

2.7.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = **, A:T = **, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.8 Aldrin and dieldrin

Aldrin and dieldrin (Figure 2.7) were formerly used as insecticides and are included in the group of chemicals known as persistent organic pollutants. Aldrin was voluntarily withdrawn from sale in New Zealand in 1985 on the basis of its toxicity and persistence in the environment. Dieldrin was deregistered in 1990 for similar reasons (C Alsford, personal communication, 1996). Aldrin is readily converted to dieldrin by plants and animals. Therefore, mainly dieldrin residues are detected in food. The structures of aldrin and dieldrin do not immediately suggest a basis for their estrogenic potential. It appears that the electronegative chlorine atoms must perform a similar role to the hydroxyl groups in 17β-estradiol.



dieldrin *aldrin*

Figure 2.7: Chemical structures of insecticides dieldrin and aldrin

2.8.1 Estrogenic activity

The estrogenicity of aldrin and dieldrin appears to be questionable with some studies reporting no estrogenic activity (Tully *et al*., 2000), while others report only very low estrogenic potency for these compounds. Based on cell proliferation assay results the EQ of dieldrin is 0.000001 (1 x 10^{-6}) (Soto *et al*., 1995) and this value will be used in the current risk assessment, again taking a worst case scenario for risk assessment purposes.

2.8.2 Occurrence in foods

Dieldrin has been detected in one of two cooked composite pumpkin samples of the 1990/1991 NZTDS at a trace concentration (0.0025 mg/kg; Vannoort *et al.*, 1995). Dieldrin has also been detected in two cucumber samples collected from New Zealand auction, gate sale or supermarket sites in 1990-91 (MAF/DoH, 1992). However, dieldrin was not detected in any food analysed in the 1987/88 and 1997/98 NZTDSs. In addition,

aldrin was not detected in any food analysed in the 1990/91 and 1997/98 NZTDSs (Vannoort *et al.*, 1995, Cressey *et al.*, 2000).

2.8.3 Exposure assessment

Given the very sporadic detection of dieldrin in New Zealand foods in the past, and the absence of any detectable amounts of dieldrin or aldrin in the 1997/98 NZTDS, the estimated daily exposures for New Zealand population groups are likely to be negligible.

2.8.4 Serum level and derived estrogenicity

Dieldrin was detected in 95 % of seurum samples from non-occupationally exposed New Zealanders, reflecting exposure at some time (Buckland *et al.,* 2001). Serum concentration ranges for three population groups of interest are shown in Table 2.8.1. This serum level, combined with relative estrogenic potency, equates to maximum estrogenicities of 2.3, 2.5 and 1.0 x 10^{-7} for adult males, females and young men as shown. In the absence of serum data for vegetarian females, their exposure is assumed to be the same as for females. With negligible current dietary intake, this estrogenicity is predicted to be a result of past exposure and would be expected to decrease over time.

Table 2.8.1: Range of dieldrin serum concentrations in non occupationally exposed New Zealanders (µg/l) (from Buckland *et al.,* 2001) and derived estrogenicity.

	Adult male	Adult female	Young male
dieldrin	0.07-0.23	0.05-0.25	0.07-0.10
Max XEQ	2.3 x 10^{-7}	2.5 x 10^{-7}	1.0 x 10^{-7}

2.8.5 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = **, A:T = **, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.9 Endosulphan

Endosulfan is a broad spectrum organochlorine insecticide currently registered for use in New Zealand (MAF, 2000). It is used to control chewing and sucking insect pests on berryfruit (blackberries, boysenberries, raspberries, blackcurrants, gooseberries and strawberries), turnips, swedes, choumoellier, rape, fodder beet, mangolds, brassicas, maize, sweetcorn, onions potatoes and tomatoes (Novachem, 1996). The structure of endosulfan (Figure 2.8) does not immediately suggest a basis for its observed estrogenic potential as there is little structural similarity to 17β-estradiol. It is likely that the electronegative chlorine atoms in endosulfan perform the role of the hydroxyl groups in 17β-estradiol.

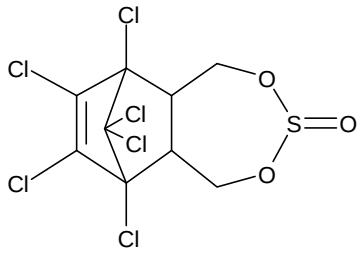


Figure 2.8: Chemical structure of the insecticide, endosulphan

2.9.1 Estrogenic activity

Technical grade endosulfan showed an estrogenic potency of 0.0001% of the potency of estradiol in the cell proliferation E-SCREEN assay using MCF-7 human breast cancer cells (EQ = 0.000001). In addition, endosulfan competed with estradiol for binding to the estrogen receptor and increased the levels of progesterone receptor and pS2 levels in MCF-7 cells (Soto *et al.*, 1995). A potency of 0.0001-0.00001% relative to estradiol was confirmed for endosulfan by Jorgensen *et al.*, (2000) by measuring the induction of the 'marker gene' pS2 in human breast cancer MCF-7 cells (EQ = 0.0000001-0.000001).
An EQ=0.000001 (1×10^{-6}) will be used for the current assessment of serum estrogenicity.

2.9.2 Occurrence in foods

Endosulfan was detected in lettuce, tomato, oil, pears, currants and raisins in the 1990/91 NZTDS. Daily intakes of 0.0541, 0.0304 and 0.0295 µg/kg bw/day were estimated for young males, adult males and children (4-6 years) respectively (Vannoort *et al.*, 1995).

Endosulfan was found in one of eight samples of tomatoes analysed in the more recent 1997/98 NZTDS at a concentration of 0.0038 mg/kg and in one of two samples of cucumbers at a concentration of 0.01 mg/kg (Cressey *et al.*, 2000).

Exposure estimates from both the 1990/91 and 1997/98 NZTDS results, and consumption of the appropriate foods, (Table 2.9.1) are indicative of a decline in exposure to endosulphan from 1990 to 1997.

2.9.3 Exposure assessment

Table 2.9.1 Estimated daily exposure to endosulfan by age–sex group (mg/day)

Year	Young male	Adult male	Adult female	Vegetarian female
1990/91[1]	0.0038	0.0024	0.0024	NR
1997/98[2]	0.00011	0.00011	0.00013	0.00019

1 Vannoort *et al.*, 1995 2 Cressey *et al.*, 2000, NR = no result

2.9.4 Actual versus theoretical serum levels

In a small Spanish study including 9 agricultural workers and two non occupationally exposed subjects, (Arrebola *et al*., 2001) the concentration of total endosulphan isomers in the non-occupationally exposed male was 7.2 µg/l and for the female was 6.6µg/l. Assuming New Zealand 1997/98 dietary intakes, the A:T ratio is approximately 170, indicative of bioaccumulation.

2.9.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity

of endosulphan for the four New Zealand populations is 4.3, 3.8, 5.9 and 8.6 x 10^{-6} µg/l for young males, adult males, females and vegetarian females respectively.

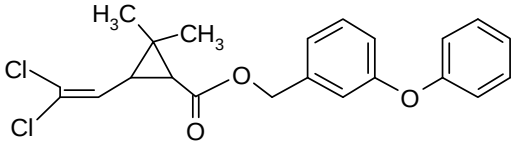
2.9.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = **, A:T = ***, Overall uncertainty = ***. See Table 2.1.2 for a description of uncertainty assignments.

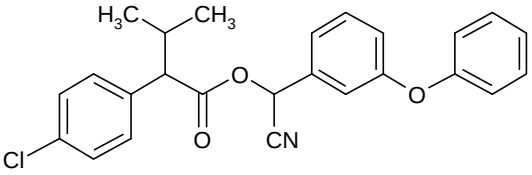
2.10 Synthetic pyrethroids

Pyrethroids are a group of synthetic derivatives of pyrethrin, the active insecticidal ingredient of the natural chrysanthemum (*Chrysanthemum cinerariaefolium*) extract known as pyrethrum. The following pyrethroids are registered for use in New Zealand : bifenthrin, cyfluthrin, cypermethrin, deltamethrin, esfenvalerate (the *S*- stereoisomer of fenvalerate which is a racemic mixture), lambda-cyhalothrin, permethrin, pyrethrins and taufluvalinate (NZFSA, 2004). For the year ending 30 June 1998, 5,300 kg of synthetic pyrethroids were used for agricultural purposes in New Zealand (Holland and Rahman, 1999). Usage has increased seven-fold in the period 1986 to 1998 (Holland and Rahman, 1999).

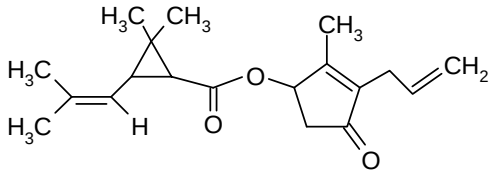
Pyrethroids appear to act by inhibiting neurotransmission and there is evidence to suggest some similarities to DDT in their mode of action, although they are structurally dissimilar (Miller and Salgado, 1985). The variety of synthetic routes starting from natural pyrethrins have resulted in a wide range of different structures. The structure-activity relationships in pyrethroids are based mainly on considerations of shape and stereochemistry rather than electronic properties (Davies, 1985).



Permethrin



Fenvalerate



Allethrin

Figure 2.9: Chemical structures of three synthetic pyrethroid insecticides

While structural analogies to 17β-estradiol are not immediately obvious, the first step in their metabolism in mammals involves ester cleavage to give carboxylic acid and alcohol

fragments (Davies, 1985) (Figure 2.10). The alcohols produced have some structural similarities to 17β-estradiol However, it is noted that these alcohols are only intermediate species in the metabolic pathway.

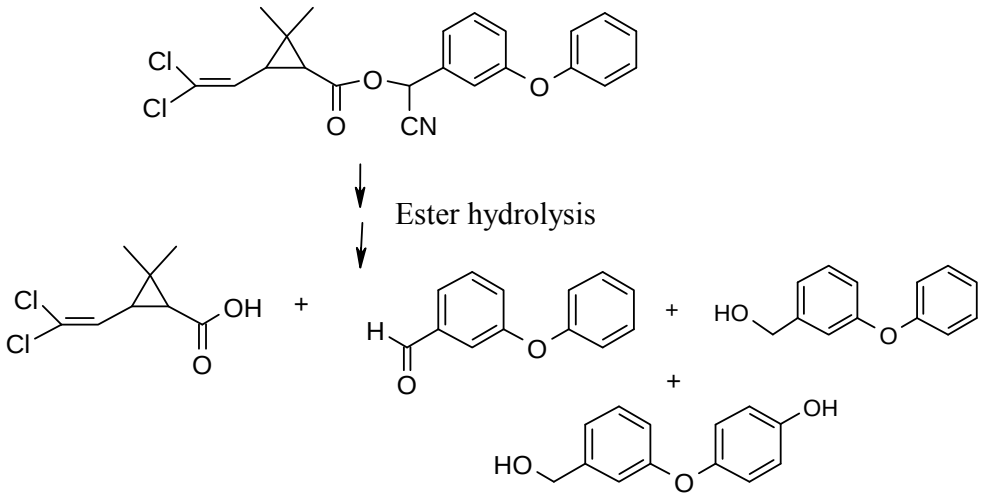


Figure 2.10: Ester hydrolysis of cypermethrin showing estrogenic metabolites (Anna McCarthy, personal communication, 2005)

2.10.1 Estrogenic activity

Measurements of the estrogenic potency of synthetic pyrethroids vary with the type of assay used (Table 2.10.1).

Table 2.10.1 Relative estrogenic potencies of synthetic pyrethroids

		***In vitro* assays**	
	Receptor binding	**Gene expression**	**Cell proliferation**
17β-estradiol	1	1	1
Pyrethrum		NE[1]	
Fenvalerate	NE[4]	NE[1], 0.0001[2],0.001[3]	**0.001[3]**
Sumithrin		0.00001[2], 0.001[3]	**0.001[3]**
Permethrin	NE[4]	NE[2,3,4]	**NE[3]**
d-trans allethrin	NE[4]	NE[2,3,4]	**0.001[3]**

NE= not estrogenic, 1= Soto *et al*., 1995, 2= Garey and Wolff, 1998 (relative to 17α-ethynlestradiol), 3= Go *et al*., 1999, 4= Saito *et al*., 2000.

Pyrethrum was reported to not be estrogenic when tested by the E-screen method (Soto *et al*., 1995). Fenvalerate and sumithrin demonstrated significant estrogenicity in gene expression assays whereas *d*-trans allethrin and permethrin did not (Garey and Wolff , 1998, Go *et al*., 1999). Fenvalerate and sumithrin also induced MCF-7 cell proliferation in a dose-dependent manner (Go *et al*., 1999).

Saito *et al*., (2000) tested the estrogenic activity of a range of pyrethroids (d-trans allethrin, cypermethrin, empenthrin, fenvalerate, imiprthrin, permethrin, d-phenothrin, and prallethrin) using three *in vitro* assays (competitive ligand-binding, luciferase reporter gene and yeast two-hybrid assays). No significant activity was observed for any pyrethroid tested using these assays.

Shaw and Chadwick (1998) demonstrated estrogenic activity for permethrin using a recombinant yeast assay. Its potency relative to 17β-estradiol was not reported.

For the purposes of the current risk assessment the estrogenic potency values determined by Go *et al*., (1999) will be utilised for synthetic pyrethroids. An upper limit EQ of 0.001 (1000×10^{-6}) will be used and applied to all synthetic pyrethroids as a conservative worst case approach. This is a simplification as obviously not all synthetic pyrethroids are equally estrogenic and emerging evidence indicates that estrogenicity of metabolites is probably more relevant from a human health perspective than estrogenicity of the parent compound (McCarthy, personal communication, 2004).

2.10.2 Occurrence in foods

The increased usage of pyrethroid pesticides in New Zealand from 1986 to 1998 is not reflected in levels reported in foods. The 1990/91 NZTDS reported detection of permethrin in one of sixteen tomato samples (Vannoort *et al*., 1995). No residues of synthetic pyrethroids were detected in any foods analysed in the 1997/98 NZTDS (Cressey *et al*., 2000). This does not mean that estrogenic metabolites were not present. Piperonyl butoxide is registered for use in New Zealand as a synergist (in this case, a compound that improves the performance of a co-applied pesticide) for pyrethrin or pyrethroid pesticides (NZFSA, 2004, Tomlin, 1994). A number of residues of piperonyl butoxide have been found in food, indicative of pyrethroid use. The ratio of pyrethroid to piperonyl butoxide ranges from 1:1 to 1:11 for formulations currently registered in New Zealand (NZFSA, 2004).

In 1990/91 the then Department of Health and the Ministry of Agriculture and Fisheries (now the Ministry of Agriculture and Forestry) carried out a survey of pesticide residues

in retail fruit and vegetables (MAF/DoH, 1992). The pyrethroid residues detected in this survey are summarised in Table 2.10.2.

Table 2.10.2: Pyrethroid residues in New Zealand fruit and vegetables, 1990/91 (Vanoort *et al.*,1995*).*

Crop	Number of samples tested	Number containing residues	Maximum (mg/kg)	Mean (mg/kg)
Fenvalerate				
Tomatoes	97	1	0.05	0.0005
Permethrin				
Tomatoes	97	9	0.08	0.006
Celery	60	2	0.11	0.003
Beans	26	1	0.19	0.007

2.10.3 Exposure assessment

Table 2.10.3 shows estimates of pyrethroid exposure for four population age-sex groups based on either of two assumptions:

1. That pyrethroids are present in foods at the levels observed in 1990/91 (MAF/DoH, 1992),
2. That pyrethroids are present in foods at the level found for piperonyl butoxide in 1997/98 (Cressey *et al*., 2000).

Table 2.10.3: Dietary exposure to synthetic pyrethroids for four population age-sex groups

Age-sex group	Intake (mg/day; assumption 1)	Intake (mg/day; assumption 2)
Young male	0.00017	**0.00546**
Adult male	0.00021	**0.0048**
Adult female	0.00021	**0.00344**
Vegetarian female	0.00020	**0.00572**

For the purposes of the current estrogenic assessment it will be assumed that all pyrethroids are estrogenic and the higher estimate for exposure will be applied.

2.10.4 Actual versus theoretical serum levels

In humans, pyrethroids are rapidly metabolized by esterases and eliminated by the kidneys (Leahey, 1985). This may account for the lack of data on human serum concentrations. In a study of 30 pest control workers in Germany, concentrations of cyfluthrin, cypermethrin and permethrin were <5µg/l (Leng *et al*., 1997). In a second study of 199 urban minority American females, *trans*-permethrin was detected in 14 (7%) of subjects at levels of <1 to 27.0 ng/l with a median of 1 ng/l. *Cis*-Permethrin was detected in 20 (10 %) of subjects at concentrations ranging from <1 to 11.4 ng/l, median = <1 ng/l (Whyatt *et al.,* 2003). Assuming a worst case serum concentration of 27 ng/l for all pyrethroids combined, and a New Zealand female dietary intake, a maximum A:T ratio of 0.02 is estimated (0.027/[0.0034x 1000/2.5]). A low A:T ratio is consistent with rapid metabolism and elimination and this value will be used for the current assessment.

2.10.5 Serum estrogenicity XEQ

Combining exposure with A:T ratio, relative estrogenic potency and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of pyrethroids for the three New Zealand populations is 2.6, 2.0, 1.9 and 3.2 x 10^{-5} µg/l for young males, adult males, females and vegetarian females respectively.

2.10.6 Uncertainty

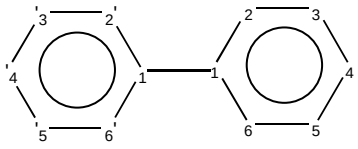
The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = ***, A:T = ***, Overall uncertainty = ***. See Table 2.1.2 for a description of uncertainty assignments.

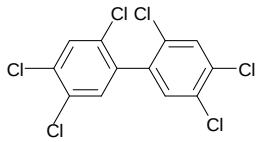
2.11 PCBs

Polychlorinated biphenyls (PCBs) are chlorinated aromatic compounds that occur as widely distributed persistent contaminants in the environment. They were formerly used in industry as electrical transformer fluids, solvent extenders, flame retardants and plasticisers, and in printing inks, paints, immersion oils and sealing liquids. Because of their persistence and accumulation in the environment, the use of PCBs in New Zealand has been prohibited, apart from under specific exemptions, since 1994. In common with most countries, New Zealand now controls disposal of these materials to ensure they are not released into the environment. PCB's have been implicated in chronic effects on reproduction, immune status, hormonal function and behaviour.

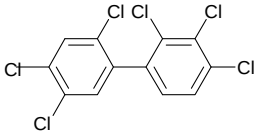
The metabolism of PCBs, mediated by cytochrome P450 in humans results in the formation of hydroxylated PCBs and several hydroxylated PCBs have estrogenic activity. Studies in laboratory animals have shown that these hydroxylated metabolites are persistent and show specific retention in blood and tissues of laboratory animals (Haraguchi *et al*., 1999).

PCBs comprise a class of 209 congeners with varying numbers of chlorine atoms attached to the basic ring structure shown below.





PCB #153



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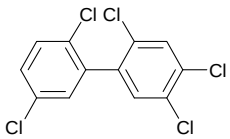


PCB #101

Figure 2.11: Basic PCB structure above, and the most prevalent PCBs found in food, below

2.11.1 Estrogenic activity

Kuiper and co-workers (1998) tested estrogenicity of hydroxylated PCBs by measuring receptor binding affinity relative to estradiol and gene expression using enhanced enzyme activity as an endpoint. They reported that in general only minimal (EQ=0.001) if any competition was detected except for 2',4',6'-trichloro-4-biphenylol and 2',3',4',5'-tetrachloro-4-biphenylol (EQ = 0.05-0.07). These same two OH-PCB compounds showed relatively strong gene expression activity relative to estradiol (EQ = 0.4-0.7) compared with the other hydroxylated PCBs (EQ=0.01).

The estrogenicity of 18 PCB congeners and 10 hydroxylated PCBs has been tested using the E-Screen assay of cell proliferation by Soto and co-workers (Soto *et al*., 1995). Four hydroxylated PCBs (where the hydroxylation occurred in the 4 position) were more estrogenic (EQ=0.0001-0.00001) than the remaining hydroxylated or non hydroxylated PCBs (EQ=0.000001).

Table 2.11.1: Relative potencies of PCBs

PCB congener	*In vitro* assays		
	Receptor binding	Gene expression	Cell proliferation
17β-estradiol	1	1	**1**
2',4',6'-trichloro-4-hydroxybiphenyl	0.05[1]	0.6-0.7[1]	**0.0001**[2]
2',3',4',5'-tetrachloro-4-hydroxybiphenyl	0.07[1] 0.00228[4]	0.4-0.7[1]	**0.00001**[2]
2'2',5-trichloro-4-hydroxybiphenyl			**0.00001**[2]
2',5'-dichloro-4-hydroxybiphenyl	0.00036[4]		**0.00001**[2]
Other PCBs[3]	0.002-0.0001[1] 0.00007[4]	0.01-0.03[1]	**0.000001**[2]

1=Kuiper *et al.*, 1998 2=Soto *et al.*, 1995 3=May include hydroxylated and non-hydroxylated PCBs. 4=Fang *et al.*,2001

Only the less estrogenic PCBs have been measured in New Zealand foods. For those PCBs, a relative estrogenicity of EQ = 0.000001 (1×10^{-6}), from cell proliferation studies, will be used for the current risk assessment. For the hydroxylated PCBs an EQ of 0.0001 is applied.

2.11.2 Occurrence in foods

PCBs are widely distributed in foods, particular high-fat foods such as meat, fish, dairy products and oils. A limited dietary survey of PCBs in New Zealand foods was carried out by the New Zealand Ministry for the Environment (MfE) (Buckland *et al.*, 1998). None of the four non hydroxylated congeners of the more estrogenic congeners identified in Table 2.11.1 were analysed in the MfE and therefore an intake estimate of these congeners is derived from the PCBs. The sum of those PCBs which were detected for each food category, has been summarised in Table 2.11.2.

Table 2.11.2: Concentrations of PCBs in New Zealand foods (Buckland *et al.*, 1998).

Food type	PCB concentration (ng /kg)
Beef Meat	58.7
Sheep meat	67.6
Pork meat	535.9
Beef fat	638.6
Sheep fat	198.4
Pork fat	194.9
Liver	104.1
Processed meats	117.5
Milk	14.9
Butter	514.1
Cheese	237.5
Ice cream/yoghurt	83.6
New Zealand fish	552.8
Imported tinned fish	2371.4
Shellfish	231.7
Poultry	27.0
Eggs	142.0
Bread	43.2
Cereals	70.1
Potatoes	52.1
Snack foods	161.9
Vegetable fats/oils	37.9

2.11.3 Exposure assessment

The MfE study for which data are reproduced in Table 2.11.2 carried out dietary exposure estimates for an average male adult (10.8 MJ/day) and a high consumer (21.5 MJ/day) adolescent male. For consistency with other dietary exposure estimates carried out as part of this chapter, the MfE data on levels of PCBs in foods has been combined with

simulated typical diets from the 1997/98 NZTDS (Cressey *et al*., 2000) to give dietary exposure estimates for young males (19-24 years), adult males, females and vegetarian females. Estimated exposure levels, expressed in terms of ng PCB/day were 90, 84, 58 and 44 ng/day for a young male, adult males, females and vegetarian females respectively. For details see Appendix 1.

2.11.4 Actual versus theoretical serum levels

The levels of non hydroxylated PCBs have been measured in the serum of non occupationally exposed New Zealanders (Buckland *et al.,* 2001). Serum from 1834 subjects was pooled into 60 samples. Serum concentrations of total PCB congeners, including non detect levels at half the limit of detection, and the serum lipid levels from which these values were derived, are shown in Table 2.11.4.

Table 2.11.3: Range of serum concentrations of total PCB congeners in non-occupationally exposed New Zealanders (µg/l) (from MfE, 2001).

Sub-population group	15-24 years	25-34 years	35-49 years	50-64 years	65+ years
Female					
Sum PCBs		1.10-1.21	1.20-1.94	1.45-2.19	1.69-2.14
Serum lipid (%w/v)[1]		0.68	0.68	0.79	0.88
Male					
Sum PCBs	0.98-1.96	1.14-1.53	1.56-1.93	1.82-2.43	2.03-2.32
Serum lipid (%w/v)[1]	0.77	0.79	0.86	0.87	0.83

Combining the intake estimates and maximum serum values from Table 2.11.4, the following A:T ratios are derived:

Table 2.11.4: Dietary intake versus serum concentration and derived serum concentration of non hydroxylated PCBs.

Sub population	Intake of total PCBs (mg/day)	Actual serum concentration-max (A) (µg/l)	Theoretical (T) serum concentration (µg/l)	A:T
Adult male	84×10^{-6}	2.43	0.025	97
Adult female	58×10^{-6}	2.19	0.023	95
Young male	90×10^{-6}	1.96	0.031	63

For the purpose of estimating estrogenicity of PCBs, a rounded A:T ratio of 100 will be assumed.

2.11.5 Serum estrogenicity XEQ

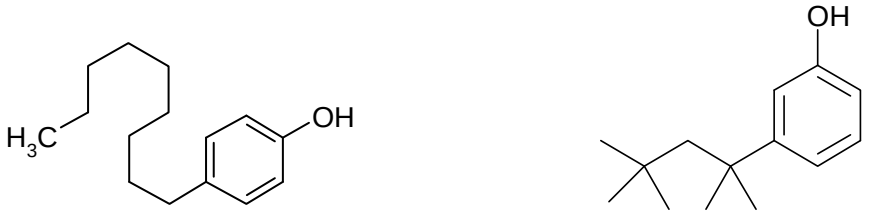
Only the less estrogenic non-hydroxylated PCBs have been determined in food and serum. Combining exposure with an A:T ratio of 100, relative estrogenic potency (1×10^{-6}) and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of non hydroxylated PCBs for the four New Zealand populations is 3.2, 2.7, 2.4 and 1.8×10^{-6} µg/l for young males, adult males, females and vegetarian females respectively. PCBs are metabolized in the mammalian system to OH-PCBs (Haraguchi *et al*., 1999) The ratio of OH-PCB in human serum has been measured in several studies with results ranging from 0.10 (Fängström *et al.,*2002), 0.11 (Sandau *et al.,* 2000) and 0.4 (Sandanger *et al.,* 2004) for subjects living in the Faroe Islands in the North Atlantic and Quebec. Assuming a worst case scenario of 0.4 for the ratio of OH-PCB to PCB, and assuming a relative estrogenic potency of 0.0001 for all OH-PCB, the contribution of estrogenicity from OH-PCB for a young male, for example, is $3.2 \times 10^{-6} \times 0.4 \times 0.0001/0.000001 = 1.3 \times 10^{-4}$ µg/l. Similarly the estimated contribution of OH-PCB to serum estrogenicity for adult males, females and vegetarian females is 1.1×10^{-4}, 1.0×10^{-4} and 0.7×10^{-4} µg/l respectively. Clearly, the estrogenicity from PCBs is potentially dominated by the hydroxylated PCBs.

2.11.6 Uncertainty

The following qualitative uncertainty assignments are associated with the exposure assessment for PCBs and thence the OH-PCBs: food concentration = **, food consumption = *, estrogenic activity = **, A:T = **, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.12 Alkyl phenols

Alkyl phenols are a group of degradation products derived from alkylphenol ethoxylates, that are non-ionic surfactants used widely in industrial detergents, paints, herbicides, household products, plastics and as process aids in pulp and paper production and textile manufacturing. Most of the ethoxylate groups are readily biodegradable, but the phenolic backbone is resistant to further breakdown. The two moieties most commonly implicated as xenoestrogens are 4-octylphenol and *p*-nonylphenol.



p-nonylphenol *4-octylphenol*

Figure 2.12: Chemical structures of the alkylphenols, *p*-nonylphenol and 4-octylphenol

2.12.1 Estrogenic activity

Estrogenicity of octyl- and nonylphenol has been well studied by all assay systems. Potency values are summarised in Table 2.12.1. The maximum estrogenic potency determined for octyl- and nonylphenol by cell proliferation assay is approximately 0.1% (EQ = 0.001) of the potency of 17β-estradiol.

Table 2.12.1 Summary of estrogenic potencies determined for octylphenol and nonylphenol.

	***In vitro* assays**			***In vivo* assays**
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1	1	**1**	1
octylphenol	0.0000036-0.00003[3]	0.001[1]	**0.001[1]**	0.00001[6]
	0.000029-0.00072[5]	0.001[2]	**0.0003[8]**	
		0.0003-0.0014[7]		
nonylphenol	0.000022-0.00005[3]	0.0001[4]	**0.000003-0.00003[8]**	0.0000036[3]
	0.001[4]			0.00001[4]
	0.000039-0.00026[5]			0.00001[6]

1= White *et al*., 1994 2= Arnold *et al*., 1996 3= Coldham *et al*., 1997
4= Shelby *et al*., 1996 5= Nagle *et al*., 1997 6= Milligan *et al*., 1998
7= Sheeler *et al.,* 2000 8=Soto *et al*., 1995.

The maximum estrogenic potency determined for octylphenol by cell proliferation assays is approximately 0.1% (EQ = 0.001) and for nonylphenol is EQ = 0.00003 (30 x 10^{-6}) of the potency of 17β-estradiol.

2.12.2 Occurrence in foods

No data are available (2005) for concentrations of octyl- or nonylphenol in New Zealand waters or foods. Levels of total 4-nonylphenol congeners ranging from <3.8 to 25 µg/kg were found in UK duplicate diet samples, with octylphenol detected in just one from the total of fifty duplicate diet samples (Fernandes *et al*., 2003),. Concentrations of alkylphenols in individual foods are not available from the UK study. However nonylphenol concentrations have been determined in 60 different foodstuffs commercially available in Germany (Guenther *et al*., 2002). Mean concentrations are shown in Table 2.12.2, along with consumption of those foods by NZ males, young males and females (Cressey *et al*., 2000, Russell *et al*.,1999).

Table 2.12.2: Mean concentration of all nonylphenol(NP) congeners in German foods (µg/kg fresh weight) (Guenther *et al*., 2002) and NZ consumption (Cressey *et al*., 2000, Russell *et al*.,1999).

Food	Mean NP conc. (µg/kg)	Consumption (g/day) YM	Male	Female	Vegetarian female
Mayonnaise	5	0.44	0.44	0.44	0.44
Sugar	6.8	13.7	13.7	13.7	13.7
Tuna	8.1	0.7	0.7	0.7	0
Butter	14.4	15.9	18.9	10.3	12.0
Lard	10.2	5.2	5.2	5.2	5.2
Spinach	1.3	1.3	1.3	1.3	1.3
Milk chocolate	14.1	2.9	2.9	1.8	2.8
Egg	1.5	21.8	16.5	13.8	22.7
Pineapple	2.6	3.6	2.1	3.6	4.3
Fresh cheese	7.5	20	29.9	18.9	32.9
Pasta	1	11.4	11.4	8.6	27.9
Apples	19.4	67.1	114.3	57.1	75
Chicken meat	3.8	35.4	35.4	27.5	0
Tea	0.1	194	80.7	255	158
Potatoes	0.6	93.2	130	51.1	111.1
Tomatoes	18.5	37.5	37.1	37.5	40
Whole milk	1.1	256	399	190	200
Wholemeal bread	1.6	30	52.5	105	126

Food	Mean NP conc. (µg/kg)	Consumption (g/day) YM	Male	Female	Vegetarian female
Beer	0.5	271	186	19.3	28.6
Coffee (brewed)	0.3	241	128	278	220
Orange juice	0.1	35	50	25.7	21.4
Freshwater fish	2020	0.08	0.08	0.08	0
Water	0.029	541	547	877	886

2.12.3 Exposure assessment

Using the concentration data reported by Guenther *et al*., (2002) and consumption of those foods by New Zealanders, from the TDS simulated diets, exposure to nonyl alkylphenols is estimated to be 4.7, 3.6, 3.0 and 3.5 µg/day for young males, adult males, females and vegetarian females respectively, with the greatest contributors to intake being apples and tomatoes. For details see Appendix 1. This is suggestive of alkylphenol ethoxylates used as emulsifiers in pesticide formulations.

2.12.4 Actual versus theoretical serum levels

There are no data on the levels of alkylphenols in the serum of New Zealanders. In a publication of methodology for the analysis of 4-nonylphenol and 4-octylphenol in human blood samples (Inoue *et al*., 2000) trace levels of alkyl phenols in the range 0.5-1.0 µg/l nonylphenol and <0.24 µg/l octylphenol were reported. Subjects are presumably Japanese, details of sample numbers are not provided. Nonyl phenol was detected in the serum of 155 (86%) umbilical cord blood samples collected from Malaysian women, with concentrations reported from <0.05 to 15 µg/l (Tan and Mohd 2003). These levels are considered high on the basis of the reported recovery information and therefore may be overestimated. Combining the intake estimates and a maximum serum value of 15 µg/l, for each population group, the following A:T ratios are derived:

Table 2.12.3: Dietary intake versus serum concentration and derived serum concentration of nonyl phenols.

Sub population	Intake of total NP (mg/day)	Actual serum concentration-max (A) (µg/l)	Theoretical (T) serum concentration (µg/l)	A:T
Young male	0.0047	15	1.42	10.7
Adult male	0.0036	15	1.09	13.9
Adult female	0.0030	15	1.20	12.6
Vegetarian female	0.0035	15	1.40	10.8

For the purpose of estimating estrogenicity of PCBs, a rounded A:T ratio of 10 will be assumed.

2.12.5 Serum estrogenicity XEQ

Since the biodegradation metabolite nonylphenol is prevalent in food and serum, whereas the octylphenol metabolite is only infrequently reported, estrogenicity from alkylphenols is best represented by data for nonylphenol (Table 2.12.2). Combining exposure with an A:T ratio of 10, relative estrogenic potency (30×10^{-6}) and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of nonylphenol for the four New Zealand populations is 6.2, 4.1, 4.6 and 5.3×10^{-4} µg/l for young males, adult males, females and vegetarian females respectively.

2.12.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = **, food consumption = *, estrogenic activity = **, A:T = ***, Overall uncertainty = ***. See Table 2.1.2 for a description of uncertainty assignments.

2.13 Bisphenols

Bisphenol is a generic term for a family of diphenylalkanes, which are currently among the leading chemicals used in the manufacture of plastics. Considerable attention with respect to estrogenicity has focused on Bisphenol A (BPA) (Figure 2.13), a primary raw material for the production of polycarbonates, epoxy resins, phenolic resins, polyesters, and polyacrylates. Epoxy-based coating are used in lacquer coatings in cans and other vessels used for foodstuffs, amongst other uses (Perez *et al.*, 1998).

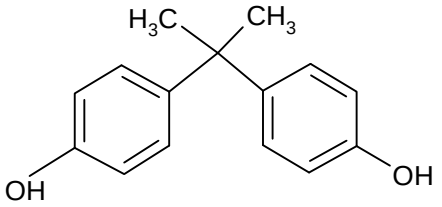


Figure 2.13: Chemical structure of bisphenol A

Structural similarities to 17β-estradiol are clear, with two distal hydroxyl groups attached to a ring structure.

2.13.1 Estrogenic activity

The estrogenic activity of BPA was first reported by Krishnan *et al.,* (1993), who observed that water autoclaved in polycarbonate flasks exhibited estrogenic activity. Since that time, estrogenic activity of bisphenol A has been demonstrated in all assay types by a number of investigators. A wide range of estrogenic equivalence ranging from EQ = 0.0018 (Fang *et al*., 2000) to 0.0000001 (Jørgensen *et al*., 2000) has been reported and is summarized in Table 2.13.1.

Table 2.13.1 Summary of estrogenic potencies determined for bisphenol-A (BPA)

	In vitro assays			*In vivo* assays
	Receptor binding	**Gene expression**	**Cell proliferation**	
17β-estradiol	1	1	**1**	1
Bisphenol A	0.0005[1]	0.0002-0.001[2]	**0.0002[1]**	0.0001[4]
	0.00006-0.0001[3]	0.00003-0.0005[5]	**0.0002-0.001[2]**	
	0.0018[7]	0.0000001-0.00001[6]	**0.00002[7]**	
	0.00056[8]	0.00005-0.000066[7]	**0.0001[8]**	

1= Krishnan *et al*., 1993 2= Steinmetz *et al.*, 1997 3= Nagle *et al.*, 1997
4= Milligan *et al.*, 1998 5= Sheeler *et al.*, 2000 6= Jørgensen *et al.*, 2000
7= Fang *et al.*, 2000 8=Perez *et al.*, 1998

For the purposes of the current risk assessment the top end (worst case) cell proliferation potency of EQ = 0.001 (1000 x 10^{-6}) will be used.

2.13.2 Occurrence in foods

There is limited data on the concentration of BPA in canned foods from the UK (Goodson *et al,* 2002) and Japan (Yoshida *et al,* 2001, Kang and Kondo, 2003). A survey of BPA concentration in canned foods has been undertaken as part of this thesis and is described in Chapter 3. Eighty different canned foods purchased from retail outlets in Christchurch, New Zealand between November 2003 and February 2004 were analysed for BPA concentration by gas chromatography/mass spectrometry. BPA was detected in all foods analysed except soft drinks. Range and mean concentrations are shown in Table 2.13.2.

Table 2.13.2: Concentration of bisphenol A measured in New Zealand canned foods (µg/kg)

Food type	Mean concentration BPA (µg/kg)	Concentration range
Tomatoes	12	<10-21
Corn	13	<10-20
Beetroot	20	13-29
Peas	8	<10-17
Apricots	5	<10
Peqches	5	<10
Pineapple	5	<10
Fruit salad	5	<10
Salmon	13	<20-24
Tuna	43	<20-109
Chicken soup	10	<20

Food type	Mean concentration BPA (µg/kg)	Concentration range
Tomato soup	11	2-16
Sauces	13	<10-21
Corned beef	46	<20-98
Other meat	10	<20
Spaghetti	5	<10
Baked beans	5	<10
Coconut cream	77	<20-192
Dessert &dairy	10	<20
Infant foods-vegetable	5	<10
Infant foods-dessert	5	<10
Soft drink	0	ND
Eurpoean olives	14	9-23

2.13.3 Exposure assessment

A slightly different approach was taken to assess exposure to BPA. Because of the aggregation of foods in the simulated diets, it is difficult to distinguish consumption of canned versus non- canned foods. Therefore, consumption data from the 1997 NNS (Russell *et al.,* 1999) that includes a much wider range of foods, was used for exposure assessments. Aggregated food descriptors were assigned a BPA concentration based on the mean concentration results determined. These foods and concentrations were combined with the 24 hour dietary recall information of the NNS survey data for each respondent for whom body weight information was available. Exposure scenarios for 4399 individuals were determined. Mean (and range) daily intakes for adult males, adult females and young males were 0.64 (0-25.4), 0.46 (0-17.9) and 0.80 (0-10.3) µg/day respectively. However, this approach does not allow for the vegetarian sub-population. For the overall assessment of estrogenicity, it is assumed the exposure for this population sub-group is the same as for adult females.

2.13.4 Actual versus theoretical serum levels

There is no data on the levels of BPA in the serum of New Zealanders. However, there have been remarkably consistent concentrations of BPA reported in four Japanese studies including normal healthy males and female subjects (Table 2.13.3).

Table 2.13.3: Serum levels of BPA reported in the literature (µg/l).

Gender	number	Concentration BPA Mean (range) µg/l	Reference
Female	12	0.33 (0-1.6)	Sajiki *et al*.,1999
Male	9	0.59 (0.38-1.0)	Sajiki *et al*.,1999
Female (pregnant)	9	0.46 (0.21-0.79)	Kuroda *et al*.,2003
Not specified (sterility patients)	21	0.46 (0.22-0.87)	Kuroda *et al*.,2003
Female	14	0.64	Takeuchi *et al*.,2002
Male	11	1.49	Takeuchi *et al*.,2002

None of these four studies cite dietary intake information but quite recently there has been an estimate of a daily intake of <0.3–7.9µg/day (median<2.0 µg/day) for Japanese pregnant women (Fujimaki *et al.,* 2004). When this intake estimate is combined with the serum data for a similar Japanese sub-population (Kuroda *et al*.,2003), A:T ratios of 3.8-0.1 are derived (Table 2.13.4). An A:T ratio of 1, reflecting a median value, is assumed for the assessment of estrogenicity.

Table 2.13.4: Dietary intake versus serum concentration of BPA for Japanese pregnant women and derived A:T ratios.

Pregnant female	Intake of BPA[1] (mg/day)	Actual serum concentration-Mean[2] (A) (µg/l)	Theoretical (T) serum concentration[3] (µg/l)	A:T
Minimum	0.0003	0.46	0.12	3.8
Maximum	0.0079	0.46	3.2	0.1
Median-approx	0.0015	0.46	0.6	0.8

1= data from Fujimaki *et al.,* 2004, 2= Kuroda *et al*.,2003, 3=intake/2.5l

2.13.5 Serum estrogenicity XEQ

Combining exposure with an A:T ratio of 1, relative estrogenic potency (1000 x 10^{-6}) and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of BPA for the three New Zealand populations is 2.8, 2.3 and 2.2 x 10^{-4} µg/l for young males, adult males and females and respectively.

2.13.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = *, food consumption = *, estrogenic activity = **, A:T = **, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.14 Butylated hydoxyanisole (BHA)

BHA is an approved antioxidant used in many countries in fats and oils and foods containing fats and oils. Use of BHA in New Zealand appears to be diminishing in favour of TBHQ (tertiary-butyl hydroquinone).

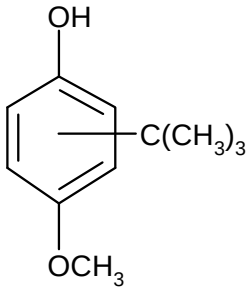


Figure 2.14: Chemical structure of the antioxidant, BHA

2.14.1 Estrogenic activity

BHA has been measured to be weakly estrogenic in both gene expression and cell proliferation assays. Soto *et al*., (1995) determined the relative proliferative potency (RPP) of BHA using MCF-7 human mammary cancer cell lines (E-Screen) as 0.00006% of the potency of estradiol (EQ = 0.0000006). This was the weakest positive response of any compound reported by Soto *et al*., (1995).

Jobling *et al.,* (1995) utilised fish and human estrogen receptors to assess the estrogenicity of a range of environmentally persistent chemicals and concluded that BHA was six or more orders of magnitude less potent than estradiol (EQ = 0.000001).

The cell proliferation based EQ of 0.0000006 (0.6×10^{-6}) will be used for the current assessment.

2.14.2 Occurrence in foods

No systematic information is available on the levels of BHA in New Zealand foods, although its usage is reported to be diminishing (Stanton, 1996).

BHA is a permitted antioxidant under the New Zealand Food Regulations 1984. BHA may be legally present in foods to a level of 100 mg/kg. If a combination of antioxidants is used the total level of antioxidants is not allowed to exceed 100 mg/kg. The Draft Australia New Zealand Food Standards Code has a more detailed list of allowed levels of BHA addition to foods, with the major relevant categories being:

- Edible oils and oil emulsions 200 mg/kg
- Instant dried mashed potato 100 mg/kg
- Chewing gum/bubble gum 200 mg/kg
- Walnut and pecan nut kernels 70 mg/kg
- Flavourings 1000 mg/kg
- Preparations of food additives 200 mg/kg

2.14.3 Exposure assessment

Addis and Hassel (1992) reported mean daily consumption of BHA to be 0.13 mg/kg body weight/day. A Dutch study reported a lower level of intake of 0.105 mg/day (0.0015 mg/kg body weight/day for a 70 kg adult; Botterweck *et al*., 2000). Intake of BHA in New Zealand is likely to be at or lower than the level reported in the Dutch Cohort Study. For the purpose of the current risk assessment a daily intake of BHA of 0.105 mg/day will be assumed for all population age sex groups.

2.14.4 Actual versus theoretical serum levels

In an Italian study, BHA was detected in 30 out of 45 human plasma samples with a mean level of 1.02 µg/l for the 30 positive samples (Bianchi *et al*., 1997). In an earlier study, nine healthy Dutch men (mean weight 74 kg) given 37mg of BHA (as a solution in corn oil) had a mean peak serum concentration of 117µg/l for BHA (Verhagen *et al*., 1989). Two scenarios for the calculation of A:T ratios for BHA from the above data are shown below (Table 2.14.1).

Table 2.14.1: Dietary intake versus serum concentration and derived A:T ratios for BHA.

Sub population	Intake of BHA (mg/day)	Actual serum concentration-max (A) (µg/l)	Theoretical (T) serum concentration (µg/l)	A:T
Dutch adult male	37	117	11212	0.01
Mixed adults	0.105[1]	1.02[2]	39	0.03

1= Botterweck *et al*., 2000 2= Bianchi *et al*., 1997

Continuing a conservative (worst case approach), an A:T ratio of 0.03 is assumed for the assessment of estrogenicity from BHA.

2.14.5 Serum estrogenicity XEQ

Combining exposure with an A:T ratio of 0.03, relative estrogenic potency (0.6×10^{-6}) and adjusting for total serum volume and molecular weight relative to estradiol, the derived serum estrogenicity of BPA for the four New Zealand populations is 0.98, 0.86, 1.1 and 1.1×10^{-6} µg/l for young males, adult males, females and vegetarian females respectively.

2.14.6 Uncertainty

The following qualitative uncertainty assignments are associated with this assessment: food concentration = **, food consumption = *, estrogenic activity = **, A:T = *, Overall uncertainty = **. See Table 2.1.2 for a description of uncertainty assignments.

2.15 Total serum estrogenicity

A summary of data for each xenoestrogen is shown in Tables 2.15.1 and 2.15.2 along with a summation of individual contributions to give an estimate of total estrogenicity from the diet for each population subgroup.

Table 2.15.1: Summary information on molecular weight (MW), estrogenic potency relative to 17β-estradiol, actual versus theoretical plasma concentration (A:T) and qualitative uncertainty of risk assessment for a range of xenoestrogens.

Xenoestrogen	MW	Estrogenic potency x 10^6	A:T ratio	Overall uncertainty
Genistein	270.2	300	0.01	*
Daidzein	254.2	100	0.005	*
Enterolactone	298.3	1	0.005	**
Enterodiol	302.4	0.1	0.005	**
Coumestrol	268.2	300	0.01	***
Quercetin	302.2	1	0.003	**
Kaempferol	286.2	70	0.001	**
Luteolin	286.2	60	0.01	***
Apigenin	270.2	150	0.01	***
Naringenin	272.3	100	0.003	**
Phloretin	274.3	25	0.006	**
Isoliquiritigenin	256.3	150	0.01	***
Zearalenone	318.4	10000	1	***
Dieldrin	380.9	1	na	**
DDT and metabolites	354.5	1	0.44	**
Endosulfan	406.9	1	170	***
Synthetic pyrethroids	391.3	1000	0.02	***
PCBs	260.0	1	100	**
OH-PCBs		100		**
Alkyl phenols	215	30	10	***
Bisphenol A	228.3	1000	1	**
BHA	180.3	0.6	0.03	**

*= least uncertain (more robust), *** = most uncertain (least robust). See Table 2.1.2 for uncertainty assignments)

The low A:T ratios of the naturally occurring xenoestrogens (of the order of 1%) are indicative of extensive metabolism by gut microflora and/or in the liver, excretion and elimination (COT, 2003). A similarly low A:T ratio is observed for the pyrethroids that are rapidly metabolized by esterases and eliminated by the kidneys (Leahey, 1985). By contrast, the synthetic estrogenic compounds show variable A:T ratios, most probably reflective of the limited serum data but also perhaps indicative of less extensive metabolism and bioaccumulation. Bioaccumulation is a feature of lipid soluble PCBs

(van Leeuwen and Younes, 2000) and explains the high A:T ratio for this group of xenoestrogens.

Where the overall uncertainty is high (***), in each case this reflects the data gaps in serum levels for that xenoestrogen, resulting in uncertainty around the corresponding A:T ratios.

Table 2.15.2: Summary information on intake and estrogenicity for a range of xenoestrogens

	Young male 19-24 yrs		Adult male 25+ yrs		Adult female 25+ yrs		Vegetarian female 19-40 yrs	
Xenoestrogen	**Intake[1] mg/day**	**XEQ µg/l**	**Intake mg/day**	**XEQ µg/l**	**Intake mg/day**	**XEQ µg/l**	**Intake mg/day**	**XEQ µg/l**
Genistein	1.4-2.5	1.4-2.5 E-03	1.5-2.7	1.4-2.4 E-03	1.0-1.4	1.2-1.7 E-03	2.9-6.7	3.5-8.2 E-03
Daidzein	1.0-1.5	1.8-2.7 E-04	1.1-1.6	1.8-2.6 E-04	0.8-0.9	1.7-2.1 E-04	3.9-5.2	8.3-11.1 E-04
Enterolactone	0.3	4.2 E-07	0.3	3.9 E-07	0.2	3.5 E-07	0.5	8.2 E-07
Enterodiol	0.2	2.5 E-08	0.1	2.0 E-08	0.1	1.8 E-08	0.2	4.1 E-08
Coumestrol	0.03	3.5 E-05	0.02	1.7 E-05	0.03	3.3 E-05	0.03	3.3 E-05
Quercetin	6.7-6.9	0.6 E-05	8.6-8.8	0.7 E-05	9.6-9.8	1.0-1.1 E-05	9.4-9.5	1.0 E-05
Kaempferol	1.8	6.2 E-05	3.5	1.1 E-04	5.0	2.0 E-04	3.0	1.2 E-04
Luteolin	0.1	2.0 E-05	0.1	1.7 E-05	0.1	3.2 E-05	0.1	2.2 E-05
Apigenin	0.3	1.6 E-04	0.3	1.4 E-04	0.5	3.3 E-04	0.3	1.9 E-04
Naringenin	2.9	2.9 E-04	2.9	2.6 E-04	1.8	2.2 E-04	1.8	2.2 E-04
Phloretin	1.6-6.8	0.8-3.5 E-04	1.1-3.7	0.5-1.7 E-04	3.1	0.5-1.9 E-04	1.0-4.7	0.6-2.8 E-04
Isoliquiritigenin	2.3	1.3 E-03	2.3	1.1 E-03	2.3	1.5 E-03	2.3	1.5 E-03
Zearalenone	0.001	3.5 E-03	0.001	2.5 E-03	0.0007	2.6 E-03	0.002	7.8 E-03
Dieldrin		1.0 E-07	-	2.3 E-07	-	2.5 E-07	-	2.5 E-07
DDT and metabolites	0.007	8.5 E-07	0.005	5.6 E-07	0.004	4.9 E-07	0.002	2.6 E-07
Endosulfan	0.0001	4.3 E-06	0.0001	3.8 E-06	0.0001	5.9 E-06	0.0002	8.7 E-06
Synthetic pyrethroids	0.005	2.6 E-05	0.005	2.0 E-05	0.003	1.9 E-05	0.006	3.2 E-05
PCBs	0.00009	3.2 E-06	0.00008	2.7 E-06	0.00006	2.4 E-06	0.00004	1.8 E-06
OH-PCBs	NA	1.3 E-04	NA	1.1 E-04	NA	1.0 E-04	NA	0.7 E-04
Alkyl phenols	0.005	6.2 E-04	0.003	4.1 E-04	0.003	4.6 E-04	0.003	5.3 E-04
Bisphenol A	0.0007	2.8 E-04	0.0006	2.3 E-04	0.0005	2.2 E-04	0.0005	2.2 E-04
BHA	0.1	9.9 E-07	0.1	8.7 E-07	0.1	1.1 E-06	0.1	1.1 E-06
Total (µg/l)		**8.1-9.5 E-03**		**6.5-7.8 E-03**		**7.1-7.8 E-03**		**15.2-20.3 E-03**
(ng/l)		**8-9**		**7-8**		**7-8**		**15-20**

1= a range is shown where intake is based on a range of concentration in the food. All other intakes are based on mean concentration data.

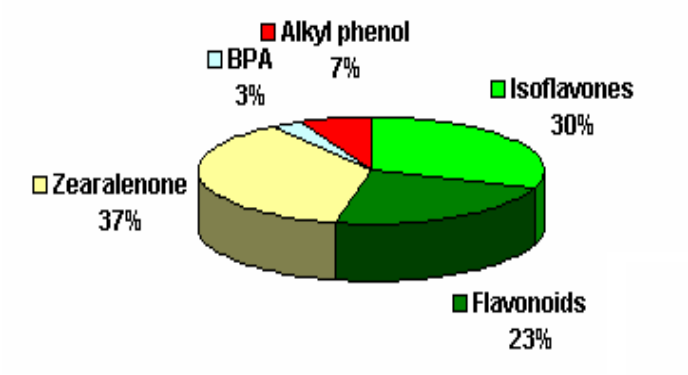
The total daily serum level of estrogenicity from food is estimated to be 8-9, 6-8, 7-8 and 15-20 ng/l for young males, adult males, females and vegetarian females respectively (Table 2.15.2).

Comparison of the total serum XEQ activity with normal serum levels of 17β-estradiol for each group gives an indication of potential pharmacological impact. The normal serum level of 17β-estradiol in human males and postmenopausal women is 10-50 ng/l (Greenspan and Gardner, 2001) and therefore it is possible that an additional contribution of 6-8 ng/l for males, 8-9 ng/l for young males, 7-8 or 15-20 ng/l for postmenopausal women might have a pharmacological effect for these sub-populations. The contribution of estrogenicity from dietary xenoestrogens for omnivorous females (7-8 ng/l) is a small proportion of natural circulating levels for a non-pregnant female (20-350 during the menstrual cycle) and therefore it is difficult to see how this level of exposure could be of health significance for this population group. (Table 2.15.3) assuming comparable transfer of xenoestrogens and 17β-estradiol across the cell membrane of target cells.

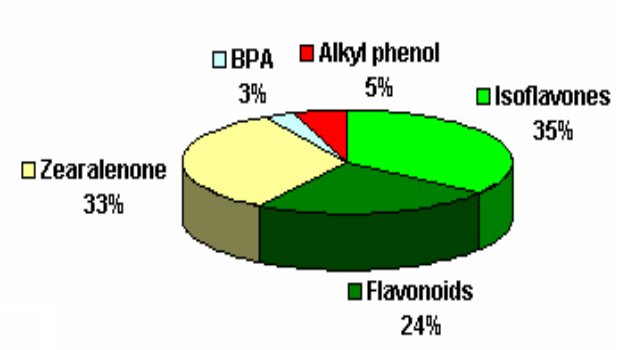
Table 2.15.3 Serum estrogenicity from the diet compared with normal circulating levels of 17 β-estradiol (ng/l) (Greenspan and Gardner, 2001, Beard and Nathanielsz, 1984).

	Adult male	Young male (>17 yr)	Adult female	Vegetarian female (19-40y)
Normal serum levels (ng/l)	10-50	10-50	Premenopausal Postmenopausal Pregnant	20-350 10-30 100-30000
Contribution from diet (ng/l)	6-8	8-9	7-8	15-20
% extra from the diet	12-80%	16-90%	Pre: 2% Post.: 23-80%	4-6%

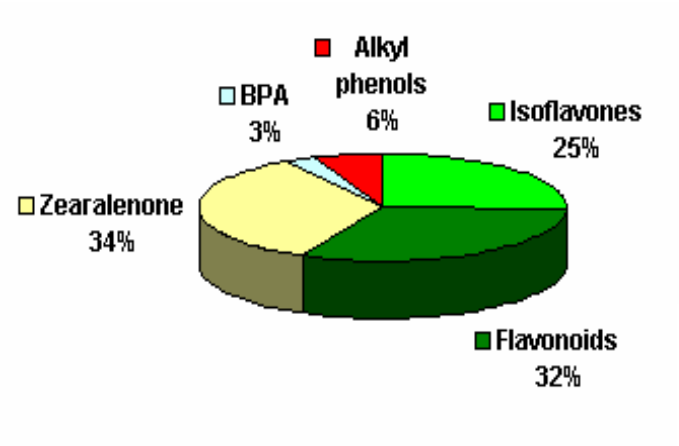
In order to assess which foods are likely to contribute most to xenoestrogen exposure from the diet, the serum estrogenicity of each xenoestrogen was assessed as a percentage of the total estrogenicity for each population group (Figure 2.15). The total estrogenicity and relative contributions of different xenoestrogens are very similar for adult males, females and young males but different for the vegetarian females.



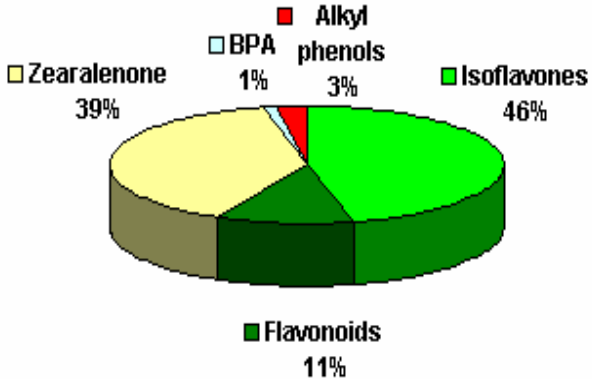
Young male



Adult male



Adult female



Vegetarian female

Figure 2.15: Distribution of estrogenicity from dietary sources for adult males (over 25 years), young males(over 25 years), adult women (over 25 years) and vegetarian women (25-40 years).

The xenoestrogens accounting for the greatest contribution to estrogenic exposure in the New Zealand diet are the contaminant mycotoxin, zearalenone (representing 33-39%), because of its very high estrogenic potency and occasional occurrence in corn based foods; the naturally occurring isoflavones (24-46%) and flavonoids (11-32%), and the synthetic compounds bisphenol A and alkylphenols (representing 1-3 and 3-7% respectively).

For the average New Zealander, the naturally occurring isoflavones, genistein and daidzein come largely from foods containing soy ingredients, and for males, from beer (Figure 2.16). The differences between absolute amount and relative contributions of estrogenicity for a vegetarian female compared with an omnivorous female are attributable to vegetarian females consuming 53 g/day of soy milk compared with no consumption for the average omnivorous female (Appendix 2). Exposure to flavonoids is dependent on consumption of specific foods; quercetin- tea and onions, phloretin – apples, kaempferol – tea and broccoli, apigenin – celery, isoliquiritigenin – licorice, and narningenin – grapefruit. Bisphenol A exposure is based on leaching from the lacquer in non acidic canned foods. Alkyl phenols are used in the manufacture of non-ionic surfactants, as additives for plastics and as pesticide excipients. However, the prevalence of use in New Zealand is unknown.

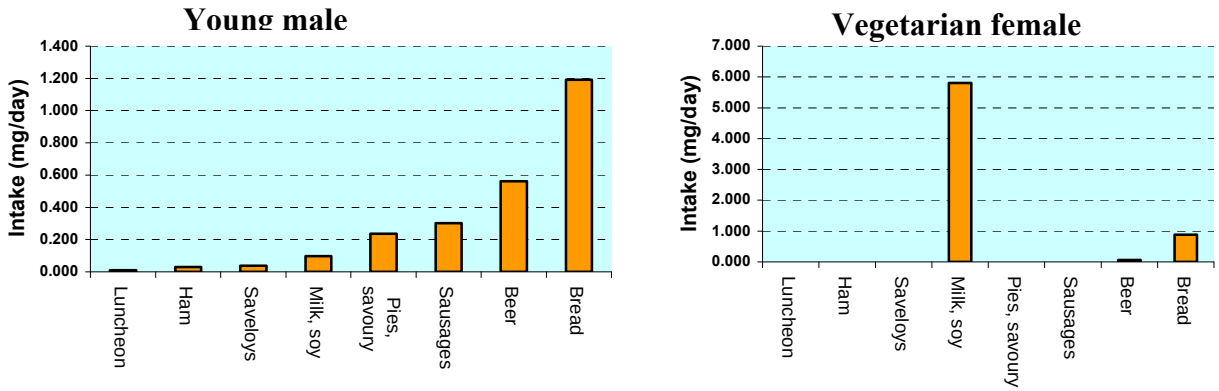


Figure 2.16: Dietary intake of geneistein for a young male and a vegetarian female

The relative contributions to dietary xenoestrogenicity calculated in this study are at variance with the findings of Shaw and McCully (2002) where the contribution from synthetic xenoestrogens was less than 1% of the contribution from phytoestrogens. This

reflects several important issues. Primarily, the model used by Shaw and McCully did not account for absorption, metabolism and excretion: the A:T modification adopted by the author, introduces these variables into the assessment and makes the model more realistic. Secondly, the current assessment draws on analytical data published since the Shaw and McCully assessment. The serum levels suggested by the current model are substantiated by literature values for 'real' serum levels of selected xenoestrogens in humans which means that the data and conclusions have *in vivo* validity.

The present assessment is still simplistic and limited by data gaps and uncertainties. For example, there is a wide range in some of the potency results, such as the synthetic pyrethroids. From the qualitative uncertainties applied, it is clearly apparent that gaps in serum data is a limitation. The most significant of these is for zearalenone because of the major contribution this mycotoxin contributes to total estrogenicity. The assumption that serum exposure levels of endosulphan in 2 Spanish subjects, is relevant to exposure in New Zealand may be debated. The resulting high A:T ratio (170) means that the contribution of endosulphan to total estrogenicity may be overestimated. Even so, endosulphan makes a very small contribution to total estrogenicity from xenoestrogens, ranking 15 /21 for contribution of individual compounds. Similarly, the serum level of 15 µg/l for nonyl phenol is for Malaysian women who will have a different exposure given the likely difference in use of this synthetic compound between Malaysia and New Zealand. Exclusion of an A:T factor (i.e.A:T =1) would reduce the contribution of alkyl phenols. In the absence of better data, a precautionary approach is justifiable (Burger, 2003). The serum concentrations applied may not reflect exposure at target cells (and therefore pharmacological effect). Indeed, Borgert *et al.,* (2003) argued that the science is insufficiently developed to allow a credible assessment of health risks to infants based on estrogenic potency or toxic effects mediated by estrogenic mechanisms. Their conclusion stems largely from the difficulty of extrapolating from estrogenic potency to possible health effects. In this current risk assessment the interpretation of risk has been calculated more simply on a comparison of cumulated estrogen equivalents for a common effect, namely cell proliferation in MCF-7 cells with circulating levels of estradiol. It is possible that the contribution from phytoestrogens is overestimated because the actual serum levels include both conjugated and parent flavonoids, but only the parent compound is estrogenic. Any contribution from estrogenic metabolites is limited to PCBs as this information is not yet available (Van de Wiele *et al.,*2005).

Despite the limitations, by taking a TEF type approach, and using the best available data, contributions from components of mixtures can be ranked and priorities for moving the science forward are identified.

It is suggestive from the model for assessing intake of xenoestrogens in food that XEQ levels in serum of New Zealanders are sufficient to have a pharmacological effect on males and postmenopausal women, but are unlikely to affect pre-menopausal females. The lack of potential impact upon premenopausal females is because of their high and incredibly variable 17β-estradiol levels during the menstrual cycle and the relatively minor contribution from xenoestrogens in the diet.

2.16 Summary`

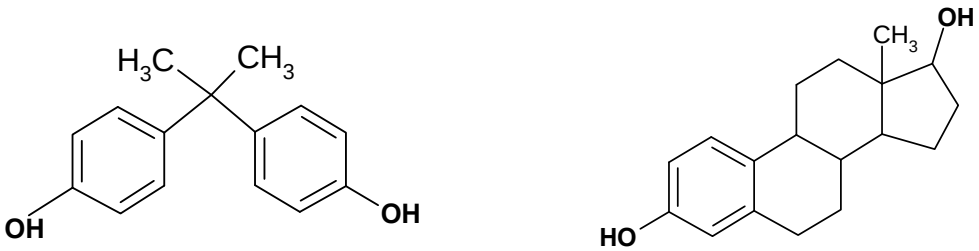
An assessment of serum estrogenicity from the daily exposure to 21 compounds (or groups of compounds) in the diet that have hormone mimicking properties has been undertaken. The total daily serum level of estrogenicity from food is estimated to be 8-9, 6-8, 7-8 and 15-20 ng/l for young males, adult males, adult females and vegetarian females respectively. Comparison with normal serum levels of 17β-estradiol is suggestive of potential pharmacological effects for males and post-menopausal women but such effects seem unlikely for premenopausal women.

Chapter 3

Exposure to bisphenol A from New Zealand canned foods.

3.1 Introduction

Bisphenol A (BPA) (Figure 3.1) is a synthetic chemical manufactured from phenol and acetone and is used in the plastics industry especially for the production of polycarbonate and epoxy resins (EC, 2003).



Bisphenol A *17β-estradiol*

Figure 3.1: Chemical structures of bisphenol A and 17β-estradiol

BPA has been shown to be estrogenic in receptor binding, gene expression, cell proliferation and *in vivo* assays. It is one of the most potent xenoestrogens that occur in the diet (Table 2.15.1) with a potency in the order of 1000 less than 17β-estradiol. BPA can enter the food chain from its use as epoxy-based lacquers of food cans and polycarbonate food containers. Food and beverage cans often have an internal polymeric coating to protect the food and prevent undesirable interactions between the metal from the can and the food. The coatings are usually resins that can withstand typical processing

conditions (e.g. 1.5h at 121°C). BPA is a starting substance used in the manufacture of epoxyphenolic resins that are the most prevalent type of can coating. BPA is also used in the manufacture of polycarbonate plastics that may be used to make baby feeding bottles, water jugs, jugs, beakers and microwave ovenware. Levels of up to about 50 µg/L BPA have been reported from migration studies using food simulants in used, but not new, polycarbonate infant feeding bottles. Migration of BPA into food from polycarbonate tableware and food storage containers is thought to be negligible (EC, 2003).

Inspection of selected canned foods showed that a variety of lacquers are used for canned foods that are available to the consumer being either white, gold, grey or clear (Figure 3.2). White, gold and grey lacquers are all epoxy based (Bill Tzimos, Technical Manager, Valspar Australia, personal communication, October 2003) and therefore potentially leach BPA into the can contents. A number of factors including pH, salt, oil and glucose have been shown to influence the migration of BPA from the lacquer to the can contents (Brotons *et al.*1995, Kang *et al.*2003).

Figure 3.2: Photograph showing variation of lacquers inside food cans

Regulatory limits of 3mg/kg and 2.5 mg/kg for BPA in canned foods have been specified by the (EU) and the Japanese government (EC, 2002, in Kang and Kondo, 2003, source reference in Japanese). The US EPA reference dose for non-carcinogenic effects from oral exposure to BPA is 0.05 mg/kg bw/day (EPA, 1993). The reference dose is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to

the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. In 2002, the European Commission Scientific Committee on Food gave a temporary Tolerable Daily Intake (TDI) of 0.01mg/kg bw/day for BPA based on a three-generation dietary rat study that showed significant reductions in adult body weight and pup body and organ weights (SCF, 2002). The TDI remains temporary because of uncertainties with the appropriateness of the rodent model and the need for more data.

There are limited data on the concentration of BPA in canned foods from the UK (Goodson *et al,* 2002) and Japan (Yoshida *et al,*2001, Kang and Kondo, 2003). Exposure estimates have been published for the UK (SCF, 2002) and US (NAP, 1999). In this chapter results for a survey of BPA in foods available to the New Zealand consumer are combined with 24-hour diet recall information to estimate over 4000 individual exposure scenarios.

3.2 Sampling

This survey was limited to BPA in canned foods and did not include possible migration from polycarbonate plasticware. The following food groups were targeted for surveillance on the basis of international results and NZ canned food consumption, from the simulated diets employed for the 1997/98 NZTDS:

Table 3.1: Canned foods targeted for analysis of BPA

Food group	Sample description
Vegetables	tomatoes, corn, beetroot, peas
Fruit	apricots, peaches, pineapple, fruit salad
Fish	salmon, tuna
Soups	chicken, tomato
Sauces	tomato, pasta sauce
Meat	based on availability
Desserts	based on availability
Canned dairy foods	condensed milk
Infant foods	based on availability
Beverages	canned soft drinks

In addition, the following factors were considered in the selection of samples for analysis:

- From observation, different brands of the same foods have different lacquers on the cans, hence brand may impact on BPA content of the food.
- From observation, different foods of the same brand come in differing lacquered cans, hence food type is a potential factor.
- High pH gives higher BPA, hence non acidic foods are more likely to have higher BPA than acidic foods (Brotons *et al*., 1995).
- Salt, vegetable oils and glucose give higher BPA levels (Kang *et al*., 2003).

The derived food list is shown in Table 3.1

Table 3.1: **Food list for the analysis of BPA in canned foods**

Food group	No. brands	No. food types	No. samples
Vegetables (tomato, corn, beetroot, peas)	4	4	16
Fruit	2	4 sweetened 4 unsweetened	8
Fish	4	2	8
Meat	4	2	8
Sauces	2	2	4
Soups	2	2	4
Spaghetti	4	1	4
Baked beans	4	1	4
Canned dairy foods	4	2	8
Infant foods	2	4	8
Soft drinks-canned	2		2
Soft drinks-bottled	2		2
Total			**76**

3.2.1 Purchase of samples

Single cans of food were purchased from major supermarkets in Christchurch in November 2003 and February 2004. Details of batch number and label information on fat and sugar content were recorded. Samples were stored at room temperature until analysis.

3.2.2 Analytical Methods

Details of the physical appearance, and area of lacquered surface of each can was recorded. Sample extraction and analysis for BPA was based on the methodology of Goodson *et al*., (2002) and is described in Chapter 8.3. One modification was made to the published methodology. For samples claiming to contain more than 1% fat (based on label claims), heptane was substituted for trimethylpentane that was found, in our hands, to be more effective at removing interfering compounds.

3.3 Quality control

The following quality assurance procedures were undertaken to assure robust results:

- Each sample was spiked with deuterated BPA. The amount of internal standard measured in the final extract provides an estimate of the extraction and cleanup losses. All results were corrected for the recovery of internal standard.
- A laboratory control sample of homogenized beetroot was analysed repeatedly during the project as a replicate to assess method variability (Table 3.2). The coefficient of variation was 8%.
- A laboratory blank was analysed with each batch of 5-8 samples to check for laboratory contamination. BPA was not detected in any of the blank samples.
- A number of samples were spiked with BPA at concentrations ranging from 6-80.4 µg/kg (Table 3.3). Spike recovery ranged from 47-112%. Despite the correction for recovery of the internal standard the reported concentrations of BPA are likely to be conservative of the actual amount.
- A number of samples were analysed in duplicate. A limit of detection (LOD) of 10µg/kg was derived for samples with less than1% fat and a LOD of 20µg/kg was derived for those samples containing greater than 1% fat (Table 3.4).

For single results, $LOD \cong 5S_R$ (Telarc, 1987)

Where S_R = standard deviation of the method and for a series of duplicates:

$$S_R = \sqrt{\frac{\Sigma (Y_1-Y_2)^2}{2p}}$$

p = number of duplicates

Table 3.3: Individual data showing within sample variability.

Replicate	Concentration BPA (µg/kg)
1	27.8
2	27.8
3	26.6
4	33.1
5	29.6
6	26.6
7	26.8
8	30.7
Mean	28.6
Stdev	2.33

Table 3.4: Recovery of BPA from samples spiked with BPA.

Food type	**% fat in sample[1]**	**Spike level (µg/kg)**	**% Recovery**
vegetable	0.2	25.1	72
fruit	0.1	25.1	67
	0.1	40.2	54,57
	0.1	80.4	89
sauce	0.2	25.1	71
spaghetti	0.1	6	60
	0.1	25.1	42
baked beans	0.3	80.4	95
meat	12.2	10	47
	20.4	80.4	112
fish	5	25.1	87

1=based on label claim

Table 3.5: Individual results for duplicate analyses of selected samples.

Food type	% fat in sample[1]	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)
vegetable	0.2	21.7	18.2
	0.5	7.7	9.6
	0.5	10.8	4.1
	0.1	17.2	14.7
fruit	0.1	5.2	5.1
	0	1.7	2.1
sauce	0.2	15	13.4
spaghetti	0.1	2.1	3.5
infant food	0.3	<0.3	<0.3
fish	7	15.1	10.5
	5	0.9	0.3
	1.9	108.5	109.8
condensed milk	8.1	3	0.3
pasta sauce	2.9	17.7	23.5
meat	NA	19.1	7.6
	20.4	92.2	103.3

1=based on label claim, NA = not available

For some food matrices, especially condensed milk, meat and fish, extracts were prone to interfering compounds that resulted in more variability and a higher limit of detection.

3.4 Concentration of BPA in NZ foods

3.4.1 Food origin

Eighty seven samples of canned foods originated from 13 different countries with the majority being products of New Zealand (34) and Australia (23). Other samples were from Thailand (6), Italy (3), USA (3), Philippines (2), Spain (2), Canada, China, Samoa, Singapore, Sth Africa and Sri Lanka (1).

3.4.2 Lacquers

All cans included at least one lacquered surface. The use of lacquers for different food types is mostly consistent. Generally, canned vegetables, sauces and vegetable based infant foods come in cans with lacquered ends and sides whereas fruits, spaghetti, baked beans, and fruit based infant foods are sold in cans with lacquered ends and tinned sides.

Meats and fish are routinely sold in grey, white or gold lacquered cans, often of a 2 piece construction. There were a few exceptions. For example three varieties of canned tomatoes came in cans with lacquered sides whilst two varieties were in cans with tinned sides. Similarly one sample of tomato sauce was in a can with lacquered sides and one sample was in a tinned can with only the ends lacquered.

3.4.3 Concentration of BPA measured in canned foods.

The application of gas chromatography- mass spectrometry (GCMS), with a deuterated internal standard, for the detection and quantification of BPA provided good selectivity. An example chromatograph is shown in Figure 3.3.

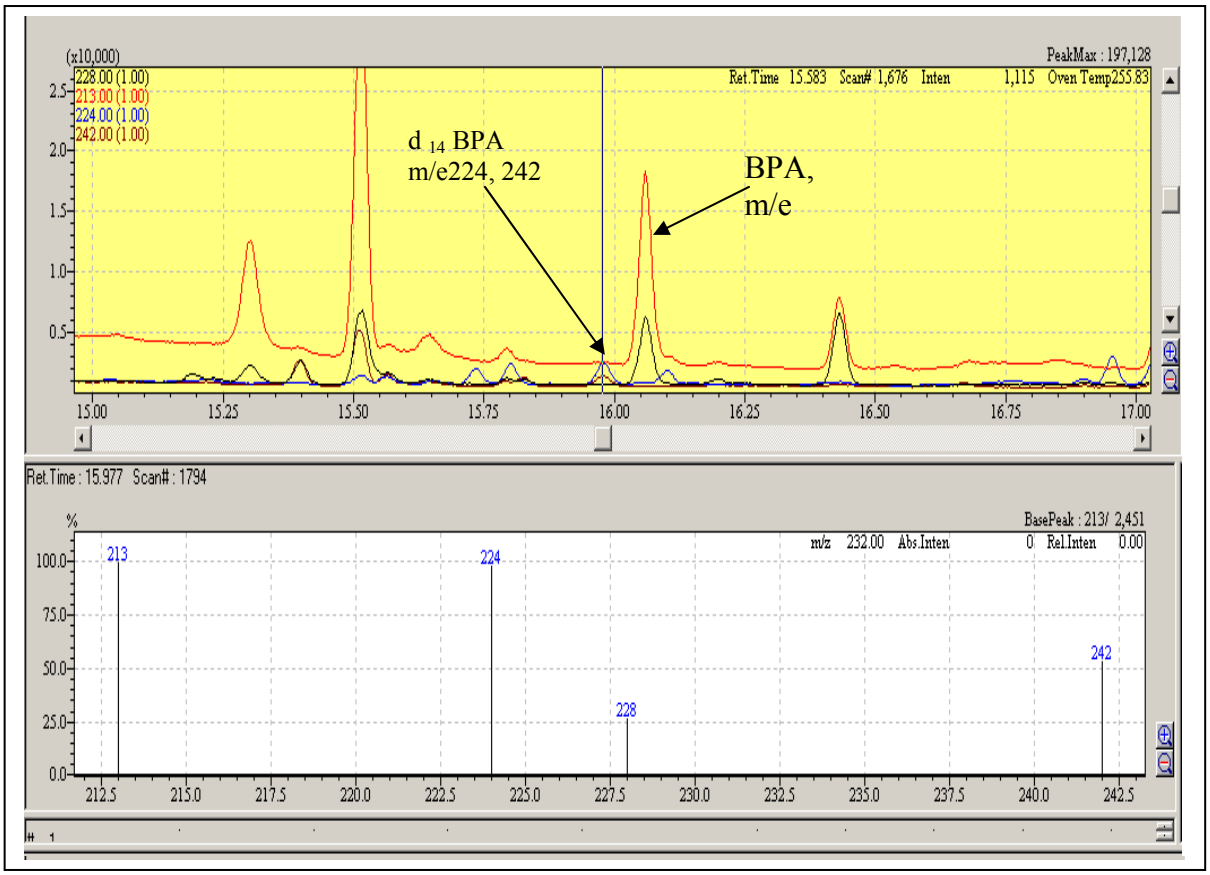


Figure 3.3: GCMS trace of an extract of beetroot showing BPA diacetyl ions m/z 228, 213 and the ions of the deuterated internal standard BPA-d_{14} diacetyl, m/z 224 and 242

A full set of analytical results for the concentration of BPA in individual samples, and mean concentrations for each food type, are listed in Table 3.5. Since all cans had at least one lacquered surface and BPA was detected in at least one sample of each food, except

the soft drinks, it is highly likely that BPA is present at some level. For this reason, "less than" values have been recorded. The limits of detection are robust and conservative being based on the variability of duplicate analyses rather than a signal: noise ratio. BPA was detected in all foods except soft drinks in which there was none detected.

Table 3.6: Concentration of BPA, area of lacqured surface, pH, % fat and % sugar in New Zealand canned foods

Food type	Country of origin	Lacquer area mm^2	pH	% fat[1]	% sugar[1]	Conc BPA µg/kg
tomatoes	NZ	30561	NR	0	2.7	16
	Australia	7695	4.2	0.1	3.8	<10
	NZ	30561	4.4	0	2.7	21
	Italy	7695	NR	0.5	3.4	<10
	NZ	30561	4.2	0.1	3.3	15
mean						**12**
corn	NZ	30561	6.5	1.8	3.3	12
	NZ	30561	6.3	1.8	3.3	14
	NZ	30561	6.3	0.2	3.5	20
	Thailand	31001	6.3	<1	3	<10
mean						**13**
beetroot	NZ	30561	4.1	0.2	10	23
	NZ	30561		0.2	10	24
	NZ	30561	4.3	0.2	8	22
	Australia	32195	4.2	0.1	11.9	13
mean						**20**
peas	NZ	30561	6.4	0.5	3	<10
	NZ	30561	6.3	0.5	3	<10
	NZ	30561	6.4	0.5	3	<10
	Australia	5280	6	0.5	4.7	17
mean						**8**
apricots	NZ	7695		<1	12.5	<10
	Australia	7695	4	0.1	12.5	<10
	NZ	7695	3.5	<1	8.2	<10
	Sth Africa	7695	3.5	0	10.1	<10
mean						**5**
peaches	Australia	7695	3.9	0.1	12.7	<10
	Australia	7695	3.8	0	12.4	<10
	Australia	7695	nr	0.1	8.7	<10
	Australia	7695	4.2	0.1	8.7	<10
mean						**5**
pineapple	Australia	5280	3.9	0.6	17.4	<10
	Philippines	5280	3.7	0	14.6	<10
	Australia	5280	3.5	0.1	12.2	<10
	Philippines	5280	NR	0	12	<10
mean						**5**
fruit salad	China	7695	3.9	0.1	15.8	<10
	Australia	7695	4.0	0	13.1	<10
	NZ	7695	3.9	0.1	10.5	<10
	Australia	7695	3.8	0.1	9.4	<10
mean						**5**
salmon	Canada	22518	6.4	7.4	<1	24
	Alaska	22518	6.4	NR	<1	<20
	NR	21378	6.4	7	0	<20

Food type	Country of origin	Lacquer area mm^2	pH	% fat[1]	% sugar[1]	Conc BPA µg/kg
	Alaska	22518	6.5	5	0	<20
mean						**13**
tuna	Thailand	20605	5.8	14	<1	27
	Thailand	20605	5.6	10	0	26
	Thailand	15177	5.9	6.5	11.3	<20
	Thailand	20605	5.8	1.9	<1	109
mean						**43**
chicken soup	Australia	30561	6.3	2.6	2	<20
	NZ	30561	5.6	2.1	3.9	<20
mean						**10**
tomato soup	Australia	7695	4.5	1	6.5	<10
	NZ	30561	4.5	0.4	4.6	16
mean						**11**
sauces	NZ	26743	3.9	0.2	21.3	14
	NZ	36719	3.8	0.2	23.6	<10
	NZ	36719	NR	0.1	20.5	11
	NZ	30561	5.2	2.9	1.9	21
mean						**13**
corned beef	Australia	28409	6	18	0	29
	Australia	28646	6	20	NR	98
	NZ	28409	6	12	<1	<20
mean						**46**
other meat	USA	23420	6.6	21	1.9	<20
	Australia	15163	6.2	12	0	<20
	NZ	28646	6.6	NA	NR	<20
mean						**10**
spaghetti	NZ	7695	4.7	0.3	3.8	<10
	NZ	7695	4.7	0.3	3.8	<10
	NZ	7695	4.6	0.1	6.8	<10
	Italy	7695	4.5	0.2	4	<10
mean						**5**
baked beans	NZ	7695	5.2	0.3	6.8	<10
	NZ	7695	5.3	0.3	6.8	<10
	NZ	7695	5.4	0.4	8.2	<10
	Italy	7695	5.6	0.4	4	<10
mean						**5**
dessert/dairy	NZ	30561	6.3	2.7	11.0	<20
	Singapore	24185	6.4	8.1	56.5	<20
mean						**10**
coconut cream	Thailand	30561	6.1	17	1.7	192
	Samoa	30561	6.2	25	1.8	<20
	Sri Lanka	30561	6	17	0	29
mean						**77**
infant food-Vegetable	Australia	16183	5.4	1.9	2.2	<10
	Australia	16183	5.5	0.3	5.7	<10
	Australia	16183	5.7	1.4	0.1	<10
	Australia	16183	5.3	0.2	3.7	<10
mean						**5**
infant food-dessert	Australia	4926	3.9	0.7	6.1	<10
	Australia	4926	4.1	0.1	11.5	<10
	Australia	16183	5.9	1.1	6.8	<10
mean						**5**
soft drinks	NZ		2.4	NA	NR	<10
	NZ		3	NA	10.2	<10
	NZ		2.4	NA	NR	<10
	NZ		3.5	NA	NR	<10
mean						**0**

Food type	Country of origin	Lacquer area mm^2	pH	% fat[1]	% sugar[1]	Conc BPA µg/kg
European olives	Spain	6840	3.6	NA	NR	<10
	Spain	30058	6.6	NA	NR	23
mean						**14**

NA=not applicable, ND = not detected, NR= no result
1=as listed on the food label

BPA was detected at a concentration above the limit of detection in 25 of the 80 samples analysed. Concentrations ranged from <10-29µg/kg except for individual samples of tuna, corned beef and coconut cream that were 109, 98 and 191µg/kg respectively (Figure 3.4). All concentrations detected were below the EU (3000µg/kg) and Japanese (2500µg/kg) migratory limits for BPA in food.

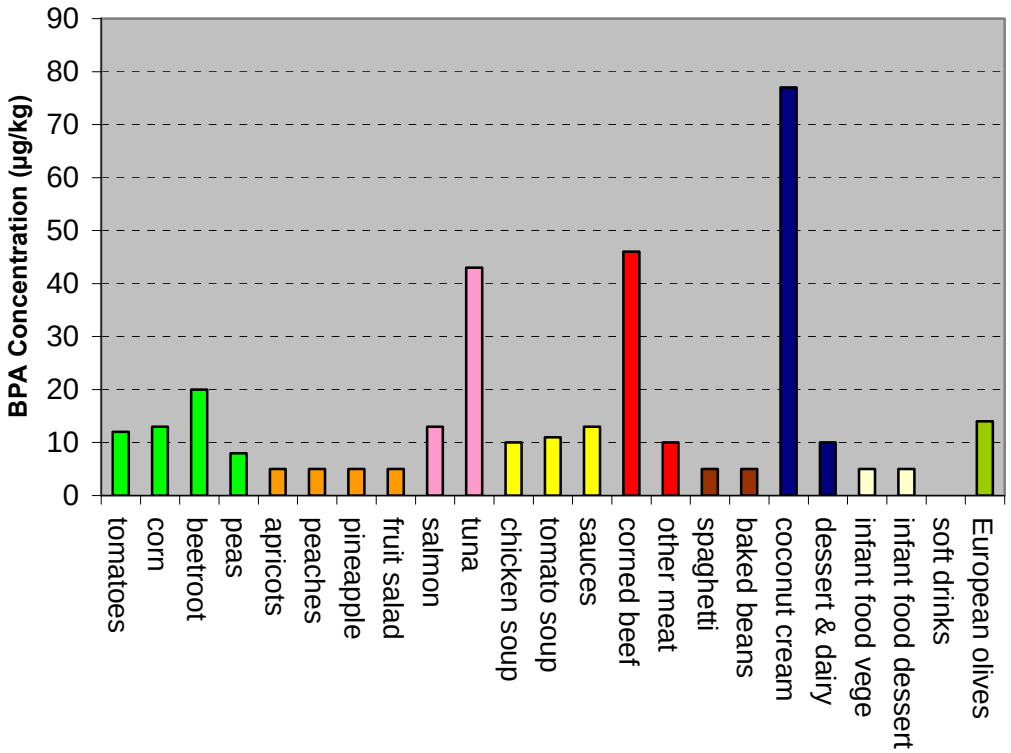


Figure 3.4: Mean concentration of BPA in different foods packed in lacquered cans (µg/kg)

3.5 Discussion

Factors influencing concentration of BPA

The area of lacquered surface, pH, sugar, oil/fat content are potentially confounding on the concentration of BPA in the can contents (Brotons *et al*., 1995, Kang *et al*., 2003). To ascertain the importance of these factors, data sets were considered where three of the four variables were constant.

Figure 3.5 shows an apparent relationship between the concentration of BPA measured relative to the area of lacquered surface when pH, fat and sugar content were the same. Not surprisingly, there was a higher concentration of BPA in the can contents when a higher proportion of the can surface was lacquered.

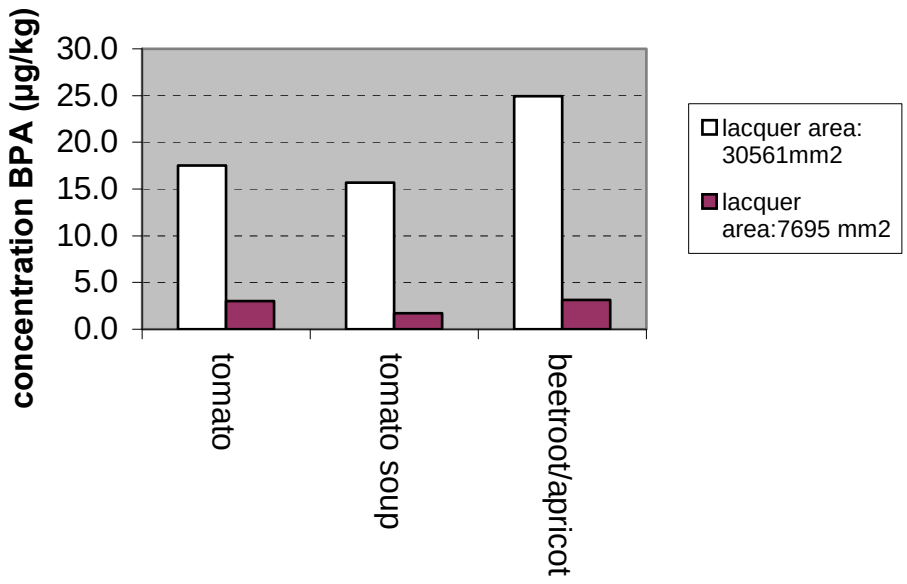


Figure 3.5: **Concentration of BPA versus area of lacquered surface for foods of similar pH, fat and sugar content.**

Contrary to the argument that BPA migration would be facilitated under alkaline conditions and, consequently, acidic foods would be less likely to leach BPA from the can lacquer than low acid foods, the mean concentration of BPA in tomatoes at pH (4.2-4.4) was almost twice that found in peas with a higher pH (6.0-6.4) for samples with comparable lacquered surface areas, fat and sugar content.

All the canned fruit samples had low levels of BPA, below the limit of detection. There was no apparent difference between the claimed sugar content and BPA concentration.

The variability and sporadically high concentration of BPA in coconut cream (192, 29, <20), corned beef (98, 29, <20) and tuna (109, 27, 26, <20) is difficult to explain but it may be related to the fat content and/or storage time. Each of these samples had >1.5% fat and BPA is fat soluble. These three products were all imported and information on the duration of storage time could not be ascertained. There is no correlation between BPA concentration, colour, area of lacquer, fat content or pH for the elevated samples. Information on the age and storage conditions of the product was not available but are potential factors. The samples with higher BPA concentration were imported foods from potentially hot locations-Thailand and Australia.

Comparison of New Zealand with International data

There is limited data available in the literature for BPA concentration in canned food and this is listed in Table 3.6. The only comprehensive overseas study was for 62 canned foods purchased from retail outlets in the UK from January to November 2000 (Goodson *et al.*, 2002).

Table 3.7 A compilation of BPA levels in canned foods reported in the current and overseas surveys.

Food type	BPA concentration range (µg/kg)		
	NZ, 2004[1]	UK, 2002[2]	Japan, 2001[3,4]
asparagus	-	-	29.8
beetroot	13-29	-	-
carrots	-	10-42	-
corn	<10-20	16	18.4-95.3
mushroom	<10	-	<10-39.5
peas	<10-17	16	-
tomatoes	<10-21	26-27	-
apricots	<10		-
fruit salad	<10	19-38	-
orange	-	-	<10
peaches	<10	-	<10
pear	-	-	<10
pineapple	<10	-	<10
salmon	<20-24	10-18	-
Other fish	<20-109	13-32	-
soups	<10-16	ND-21	-

Food type	BPA concentration range (µg/kg)		
	NZ, 2004[1]	UK, 2002[2]	Japan, 2001[3,4]
sauces	<10-21	-	-
corned beef	<20-98	59-70	-
other meat	<20	16-422	-
spaghetti	<10	ND-38	-
baked beans	<10	9-14	-
coconut cream	<20-192	-	-
dessert	<20	ND-13	-
milk, condensed milk	<20	11-14	21-43
infant foods-vegetable	<10	-	-
infant foods-dessert	<10	-	-
soft drinks,cider,lager	ND	ND	-
European olives	9-23	-	-
Total	25/80 positive	38/62	11/17

- = data not available, 1= this survey, 2= Goodson et al.,2002, 3 = Yoshida et al., 2001, 4 = Kang and Kondo, 2003.

The levels found in New Zealand foods are low and comparable with international results for fruits, vegetables, soups, corned beef, spaghetti, baked beans, condensed milk and soft drinks (Goodson *et al,.* 2002, Yoshida *et al.,*2001, Kang and Kondo, 2003). Canned ham, that had high levels in the UK study, was not found on New Zealand supermarket shelves and levels of BPA in New Zealand meat products that might be comparable, such as tongue and luncheon meat, were below the limit of quantitation (LOQ) in the current survey. The high level of BPA found in individual samples of tuna and coconut cream have not previously been reported. The absence of any detectable BPA in canned soft drinks is consistent with the findings of Goodson *et al*. (2002) and migration studies reported by Howe *et al.* (1998). The lacquered surface of beverage cans are silver coloured rather than grey, white or gold suggesting a different lacquer system that may account for the non detection of BPA in the limited number of beverages analysed in this study.

Estimated dietary exposure to BPA

Aggregated food descriptors from the 1997 National Nutrition Survey (Russell *et al.,* 1999) were assigned a BPA concentration based on the concentration results determined in this survey. A total of 1046 food descriptors were used. These foods and concentrations were combined with 24 hour dietary recall information for 4399 individual consumers for whom body weight information was available, to generate daily exposure scenarios for BPA.

Since all cans, with the exception of soft drinks, had at least one lacquered surface, there was potential for BPA to be present in the can contents. Where BPA was not detected in a sample or a food group, a value of half the limit of detection was assigned, in line with international recommendations (WHO, 1995). The exception was soft drinks where there was no evidence of BPA. Soft drinks were not included in foods contributing to BPA exposure.

For foods that had not been analysed in this survey, such as asparagus, plums and mackerel, a concentration value for BPA was approximated from similar foods. For fruits and vegetables, a mean of all vegetable results (13 µg/kg) and a mean of all fruit results (5 µg/kg) was determined and assigned to the foods outside this survey. For non specified canned fish , the mean concentration of BPA for combined tuna and salmon results was applied (28 µg/kg). Where a canned food might be included in a recipe, such as canned tomatoes in spaghetti bolognaise, a proportion of the ingredient in the recipe was approximated.

The mean and percentile dietary exposure estimates derived by combining mean BPA levels from the current survey with 24-hour dietary recall information are summarized in Table 3.8 and Figure 3.6. The mean body weight and exposure to BPA for these 4399 individuals was 74.8 kg and 0.008 µg/kg bw/day respectively. Assuming this sample of individuals is representative of the New Zealand population, the mean per capita exposure to BPA from the consumption of canned food is 0.008 µg/kg bw/day and the level of exposure for the 97.5th percentile of the population is 0.063 µg/kg bw/day . From these scenarios, however, most people (59%) are not exposed to BPA because they do not consume canned foods. The highest level of exposure for one individual was 0.29 µg/kg bw/day, well below the *t*-TDI of 10 µg/kg bw/day.

Exposure was calculated both with and without a contribution of 50% LOQ for soft drinks. The inclusion of soft drinks doubled the mean exposure. But this is considered an over-estimate because we have no evidence that BPA leaches from bottles or cans into beverages. Thus, exposures excluding a contribution from canned or bottled beverages beverages, have been presented as the most realistic assessment.

Table 3.8: Mean and percentile dietary exposure estimates for BPA based on concentration data derived from this survey and 24 hr dietary recall information of food consumption for 4399 New Zealand adult consumers (Russell *et al.,* 1999).

	Estimated Dietary Exposure	
	µg/day	µg/kg body weight/day
Mean	0.57	0.0083
Median	0.00	0.00
Range	0-25	0-0.29
95th percentile	3.1	0.041
97.5th percentile	4.7	0.063
99th percentile	6.9	0.092
***t*-TDI**		**10**

t-TDI = temporary Tolerable Daily Intake

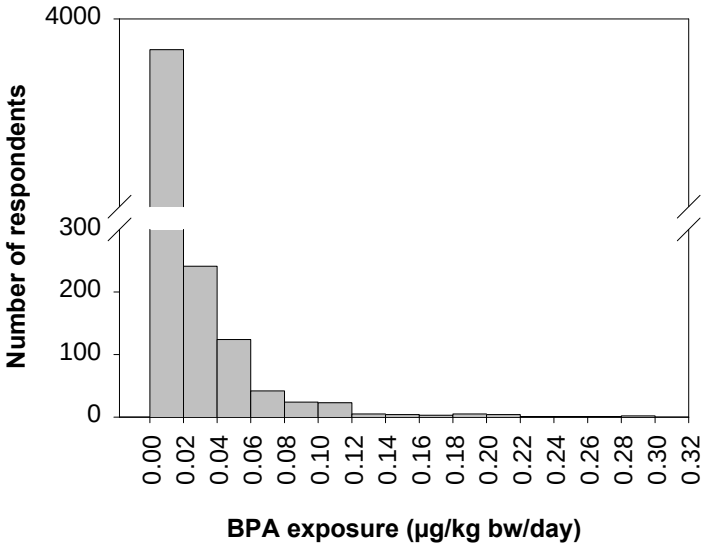


Figure 3.6: Distribution of exposure to BPA (4399 scenarios derived from the food consumption information of the 1997 NNS, Russell *et al*. 1999)

Foods contributing to dietary exposure

Exposure to BPA is mainly through consumption of canned sauces followed by coconut cream, corned beef, canned tuna, tomato soup and canned tomatoes (Table 3.8). Exposure to BPA for the individual with the highest BPA exposure scenario (0.28 µg/kg bw/day) was from the consumption of coconut cream. The lowest exposure scenario (above zero) was from the consumption of 1.1g of chilli sauce.

Table 3.9: Contribution of foods included in the current study to dietary exposure to BPA.

Food	% contribution
Sauces, canned	30.5
Coconut cream, canned	9.0
Beef, corned, canned	7.9
Tuna, canned	7.8
Soup, tomato, canned	6.4
Tomatoes, canned	6.2
Beetroot, canned	3.8
Spaghetti, canned	3.6
Corn, canned	3.3
Salmon, canned	3.3
Peaches, canned	3.3
Beans, baked, canned	3.2
Fish, other, canned	3.0
Fruit, other, canned	1.8
Vegetable, other, canned	1.7
Fruit salad, canned	1.6
Pineapple, canned	1.4
Soup, chicken, canned	1.0
Apricots, canned	0.8
Peas, canned	0.3
Other meat, canned	0.2
Dessert & dairy, canned	0.1
Other	0.0

Comparison with previous estimates of dietary exposure

The limited comparative exposure estimates for BPA are shown in Table 3.10. An intake of 0.37 μg/kg bw/day BPA has been estimated for a 60 kg adult consuming 1.05 kg of canned food per day (SCF, 2002). This estimate assumes a high level of consumption, the 97.5th percentile consumer, and a mean contaminant level of 21.2 μg/kg across the 62 foods reported in the UK study of Goodson *et al.* (2002). Therefore it is an overestimate of exposure for the average consumer.

A potential daily intake of 6.3μg/day of BPA from food cans has been derived for the US consumer, equivalent to 0.105 and 0.084 μg/kg bw/day for a 60 or 75 kg adult respectively (NAP, 1999). This estimate was based on an average migration concentration of 0.37μg/kg from extraction studies using food-stimulating solvents, along with a factor to account for the proportion of an individual's daily diet likely to be in

contact with lacquer from a food can (Howe e*t al*., 1998) and a daily food consumption of 2.89kg.

Table 3.10: Comparison of NZ and overseas exposure estimates for BPA

	NZ 2004[1]	UK, 2002[1]	USA, 1998[2]
µg/day	4.7	22.2	6.3
µg/kg bw/day for 60kg bw	0.078	0.37	0.105
µg/kg bw/day for 75kg bw	0.063	0.30	0.084

1 97.5th percentile consumer, UK = SCF, 2002
2 mean consumption, NAP,1999.

From over 4000 exposure scenarios generated in this study there was no individual who exceeded the European Commission SCF *t*-TDI for BPA. Most individual scenarios resulted in zero exposure to BPA from canned food. The results of this survey suggest that at the current level of understanding, the concentrations of BPA identified in canned foods are unlikely to be of concern to adult health, and there is no reason for consumers to change their consumption patterns as a result of these findings. This health implication is limited to adults until consumption information for other population groups is available.

This survey provides the first data on the concentration of BPA in New Zealand canned foods. The resulting estimate of exposure to BPA from canned foods is considered the most robust of any international estimates available at this time because it is derived from concentration values for individual foods, in combination with over 4000 individual exposure scenarios from 24-hour dietary records.

3.5 Summary

Exposure to bisphenol A (BPA) from the consumption of canned and bottled food has been determined for New Zealand adults. Eighty different canned foods purchased from retail outlets in Christchurch, New Zealand between November 2003 and February 2004 were analysed for BPA concentration by gas chromatography/mass spectrometry. BPA was detected in all foods analysed except soft drinks at concentrations ranging from <10-29µg/kg, except for individual samples of tuna, corned beef and coconut cream that were 109, 98 and 191µg/kg respectively. The limit of detection, based on the variability of

duplicate results, was <10µg/kg for foods of <1% fat content and <20 µg/kg for foods containing >1% fat. Mean concentration data was combined with 24-hour dietary recall information for 4399 individual consumers. Mean and maximum exposures were 0.008 and 0.29 µg/kg bw/day respectively, well below the *t*-TDI of 10µg/kg bw/day given by the European Commission in 2002. The results of this survey suggest that the levels of BPA identified in canned foods are unlikely to be of concern to adult health, and there is no reason for consumers to change their consumption patterns as a result of these findings.

Chapter 4

The role of gut microflora on the bioavailability of isoflavones

4.1 Introduction

Only 0.5-1% of ingested isoflavones are measured in serum (Chapter 2.2). Gut microflora have been implicated as having a role in the bioavailability of isoflavones from soy containing foods (Hendrich *et al.,* 1998, Rowland *et al*., 1999, Lampe *et al.,* 1998). This chapter includes an extensive review of the physiology of the human gut, the identity and distribution of gut microflora, and microfloral metabolism of isoflavones. With this background, the hypothesis that gut microflora activity accounts for the difference between estimated and measured serum levels from dietary intake of isoflavones is tested through experimental studies of the degradation of soy isoflavones by the gut microflora of 9 New Zealanders.

4.1.1 Physiology of the Human Gut and its Microflora

The chemical and physical nature of the gastrointestinal tract is diverse, thus creating myriad environments that select microflora populations by their habitat requirements (Tannock, 1995) (Figure 4.1). The stomach has a pH of approximately 2.0 (Tannock, 1995, Drasar and Barrow, 1985), and therefore acts as a barrier to microbes entering via the mouth. The chyme leaving the stomach is neutralized by bile and pancreatic secretions. The small intestine is subdivided into the duodenum (approximately 0.25 m), jejunum (approximately 2.5 m), and ileum (approximately 3.5 m) with the majority of digestion and absorption occurring in the jejunum (Johnson and McCormack, 1994).

Food takes 3 to 6 hours to move through the small intestine, depending on its consistency (Xu *et al*.,1995). The large intestine (colon) is 1.5 to 1.8 m long (Spence and Mason, 1992) and digestive residue transit time is at least 12 hours before elimination through the rectum and anus (Lamb *et al.*, 1991). The transition of structural and functional characteristics through the different intestinal regions result in ecologic changes in gut microflora that favor colonization by particular microflora communities (Drasar and Barrow, 1985).

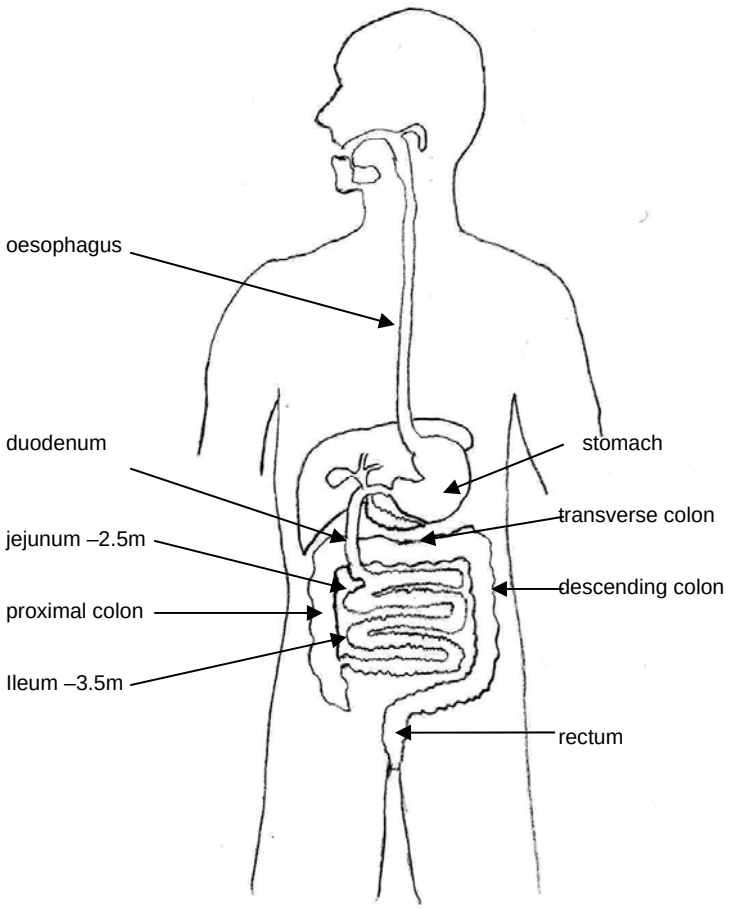


Figure 4.1: Schematic diagram of the human gastrointestinal tract. Adapted from Shaw IC, Is it safe to eat? Springer, Berlin (2004) by permission of the author.

Studies on faecal bacteria show that once the flora are established in an individual they change little, even in response to dietary change (Drasar and Barrow, 1985, Simon and Gorbach, 1984, 1986, Carman *et al.,*1993, Edwards, 1994, Nord and Kager, 1984, Hentges,1980). Despite the apparent consistency of an individual's microfloral population, dietary change may induce significant changes in microfloral metabolic

activity (Rowland, 1986, Simon and Gorbach, 1984, 1986, Carman *et al.,*1993, Edwards, 1994, Hentges,1980). The influence of diet on the metabolism of isoflavones has been observed (Kelly *et al*.,1993, Setchell *et al.,* 1984, Lampe *et al*., 1998).

Intestinal bacteria can be divided into three groups: (1) lactic acid bacteria comprising *Bifidobacterium*, *Lactobacillus,* and *Streptococcus;* (2) the obligate anaerobes including Bacteroidaceae (encompassing the genus *Bacteroides*), *Eubacterium*, *Veillonella*, and *Clostridium;* (3) the aerobes (most of which are facultative anaerobes) including *Staphylococcus*, *Bacillus*, *Corynebacterium*, *Pseudomonas* and yeasts, and Enterobacteriaceae (including pathogenic genera such as *Escherichia*, *Klebsiella,* and *Enterobacter*) (Mitsuoka *et al.,* 1992).

4.1.2 Distribution of the gut microflora

Despite there being inter-individual variability in gut microfloral populations, a general pattern of microfloral colonization can be observed along the gastrointestinal tract in humans. Consolidated data from numerous works are summarized in Table 4.1 (Drasar and Barrow, 1985, Hawkesworth *et al.,*1971, Simon and Gorbach, 1984,1986, Orrhage and Nord,2000, Mitsuoka, 1992, Bernhardt and Knoke,1989, Thadepalli *et al.,*1979, Goldin,1990).

Table 4.1: Distribution of major microflora in the human gastrointestinal tract. (Drasar and Barrow, 1985, Hawkesworth *et al.*,1971, Simon and Gorbach, 1984, 1986,Orrhage and Nord, 2000, Mitsuoka, 1992, Bernhardt and Knoke, 1989, Thadepalli *et al*.,1979, Goldin, 1990).(compiled by N. Turner).

Stomach			Log count of bacteria (CFU/ml or CFU/g)											
	Microorganisms	**Count**	1	2	3	4	5	6	7	8	9	10	11	12
Total count		$0\text{-}10^4$	■	■	■	■								
Facultative anaerobes	*Streptococcus*	$0\text{-}10^3$	■	■	■									
	Enterococcus	Rare												
	Staphylococcus	$0\text{-}10^2$	■	■										
	Enterobacteria	$0\text{-}10^2$	■	■										
	Yeasts	$0\text{-}10^2$	■	■										
	Lactobacillus	$0\text{-}10^3$	■	■	■									
Obligate anaerobes	*Bifidobacterium*	$0\text{-}10^2$	■	■										
	Bacteroides	Rare												
	Fusobacterium	$0\text{-}10^2$	■	■										
	Eubacterium	Rare												
	Veillonella	$0\text{-}10^2$	■	■										
	Clostridium	Rare												
	Peptostreptococcus	$0\text{-}10^3$	■	■	■									
	Prevotella	$0\text{-}10^2$	■	■										

Duodenum/Jejunum.			Log count of bacteria (CFU/ml or CFU/g)											
	Microorganisms	**Count**	1	2	3	4	5	6	7	8	9	10	11	12
Total count		$0\text{-}10^5$	■	■	■	■	■							
Facultative anaerobes	*Streptococcus*	$0\text{-}10^4$	■	■	■	■								
	Enterococcus	$0\text{-}10^2$	■	■										
	Staphylococcus	$0\text{-}10^3$	■	■	■									
	Enterobacteria	$0\text{-}10^3$	■	■	■									
	Yeasts	$0\text{-}10^2$	■	■										
	Lactobacillus	$0\text{-}10^4$	■	■	■	■								
Obligate anaerobes	*Bifidobacterium*	$0\text{-}10^4$	■	■	■	■								
	Bacteroides	$0\text{-}10^3$	■	■	■									
	Fusobacterium	$0\text{-}10^3$	■	■	■									
	Eubacterium	Rare												
	Veillonella	$0\text{-}10^3$	■	■	■									
	Clostridium	Rare												
	Peptostreptococcus	$0\text{-}10^3$	■	■	■									
	Prevotella	$10^2\text{-}10^4$			■	■								

Ileum (lower small intestine)			Log count of bacteria (CFU/ml or CFU/g)											
	Microorganisms	**Count**	1	2	3	4	5	6	7	8	9	10	11	12
Total count		$10^4\text{-}10^8$				■	■	■	■					
Facultative anaerobes	*Streptococcus*	$10^2\text{-}10^4$			■	■								
	Enterococcus	$10^2\text{-}10^4$			■	■								
	Staphylococcus	$10^2\text{-}10^5$			■	■	■							
	Enterobacteria	$10^2\text{-}10^7$			■	■	■	■	■					
	Yeasts	$10^2\text{-}10^4$			■	■								
	Lactobacillus	$0\text{-}10^5$	■	■	■	■	■							

Obligate anaerobes	*Bifidobacterium*	10^3-10^9				■	■	■	■	■	■				
	Bacteroides	10^3-10^7				■	■	■	■						
	Fusobacterium	10^3-10^4				■									
	Eubacterium	Rare													
	Veillonella	0-10^4	■	■	■	■									
	Clostridium	0-10^4	■	■	■	■									
	Peptostreptococcus	10^2-10^6			■	■	■	■							
	Prevotella	10^3-10^4				■									

Colon			Log count of bacteria (CFU/ml or CFU/g)											
	Microorganisms	**Count**	1	2	3	4	5	6	7	8	9	10	11	12
Total count		10^{10}-10^{12}										■	■	
Facultative anaerobes	*Streptococcus*	10^3-10^5				■	■							
	Enterococcus	10^5-10^{10}						■	■	■	■	■		
	Staphylococcus	10^4-10^6					■	■						
	Enterobacteria	10^4-10^{10}					■	■	■	■	■	■		
	Yeasts	0-10^5	■	■	■	■	■							
	Lactobacillus	10^6-10^8						■	■					
Obligate anaerobes	*Bifidobacterium*	10^7-10^{11}							■	■	■	■		
	Bacteroides	10^8-10^{12}								■	■	■	■	
	Fusobacterium	10^6-10^8						■	■					
	Eubacterium	10^9-10^{12}									■	■	■	
	Veillonella	10^3-10^6			■	■	■							
	Clostridium	0-10^9	■	■	■	■	■	■	■	■				
	Peptostreptococcus	10^{10}-10^{12}										■	■	
	Prevotella	10^4-10^5				■								

The micro-organisms of the stomach are a reflection of the oral flora that are able to survive the acidic environment (Drasar and Barrow, 1985). They are mainly gram-positive facultative anaerobes such as *Streptococcus*, *Staphylococcus,* and *Lactobacillus* (Goldin, 1990). The duodenal flora also reflect swallowed bacteria and those that survived the stomach's acidic conditions (McDonald, 1984). The duodenum is a region in constant change. The low pH of the upper intestinal contents owing to gastric acid is adjusted to pH 6 to 7 by the alkaline bile and pancreatic juices. However pH is a strong enough influence that only acid-tolerant bacteria grow (Tannock, 1995, Gorbach and Goldin, 1992). Bile and pancreatic fluids also provide sources of nutrients. The flow rate of the intestinal contents, which is at its maximum at the top of the small intestine, selects for limited establishment of microflora able to best survive in the transient environment (Tannock, 1995, Drasar and Barrow,1985). The predominant duodenal flora are gram-positive facultative anaerobes. Coliforms such as *E coli* and *Klebsiella* are also present at a low count, but these are probably transients (Thadepalli *et al*.,1979, Goldin, 1990).

The jejunal flora is similar to that of the duodenum in that it is dominated by the transient *Lactobacillus* and *Streptococcus* (Tannock, 1995). Although the total bacterial numbers may reach 10^5/mL of intestinal contents, sterility in this area is not unheard of (Justesen *et al.,* 1984a,b). As with the duodenum, strict anaerobes may also be found in low numbers (Gorbach and Goldin, 1992).

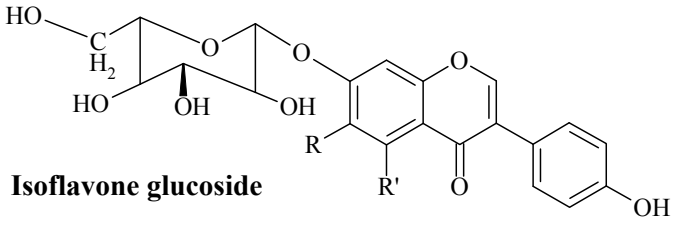
The ileum has a great variability in microfloral distribution as it represents a transition in microflora from the jejunum to colon. The upper region has similar microflora to the jejunum, but increasing distance from the jejunum coincides with a general increase in bacterial numbers; by the end of the small intestine, therefore, the bacterial species are similar to the colon, just in smaller numbers (McDonald, 1984). The most common genera found in the distal ileum are *Bifidobacterium* and *Bacteroides* (Orrhage and Nord, 2000, Goldin, 1990). *Lactobacillus*, *Streptococcus* (including *Enterococcus*), *Clostridium,* and members of the Enterobacteriaceae (such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia*) are also prominent (Tannock, 1995, Orrhage and Nord, 2000). Low oxygen tension favors growth of obligate anaerobes; however 50% of bacteria present may still be facultative anaerobes (Simon and Gorbach, 1986, McDonald,1984).

The colon has a large bacterial population (Gorbach and Goldin, 1992), is more anaerobic and has a much reduced flow rate than the small intestine. The total concentration of bacteria in the colonic contents (10^{11}–10^{12} colony-forming units [cfu]/mL) approaches the theoretic limit that can fit into a given mass (Gorbach and Goldin, 1992). Greater than 400 species have been isolated from this region, with the obligate outnumbering facultative anaerobes by 1000:1 (Nord and Kager, 1984). *Bacteroides*, *Bifidobacterium,* and *Eubacterium* dominate the obligate anaerobes; however, the Enterobacteria and *Enterococcus* are still present in high numbers (Orrhage and Nord, 2000). The main method for analyzing the colonic flora has been through studies of the feces in which nearly one-third of faecal dry weight consists of viable bacteria (Simon and Gorbach, 1984). Opinions on the validity of using feces as an indicator of colon microflora differ (Drasar and Barrow, 1985, Moore and Moore, 1995, Kasper,1998).

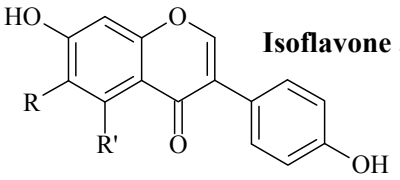
4.1.3 Metabolism of isoflavones by microflora

4.1.3.1 Releasing the aglycone

The isoflavones genistein and daidzein (Figure 4.2) often occur in nature as glycosides (Goldin, 1990). The first step in their metabolism is loss of the sugar to release the aglycone (Figure 4.3) (Scalbert and Williamson, 2000, Xu *et al*., 1995, Rowland *et al.,* 1999, Kim *et al*., 1998). Although a portion of this metabolism may be attributed to nonbacterial enzymes, in the intestinal tract, this is minor in comparison with the activity of gut microflora (Davenport, 1982).



Isoflavone glucoside



Isoflavone aglycone

Compound		R	R’
Glucosides	Daidzin	H	H
	Genistin	H	OH
	Glycitin	OCH_3	H
Aglycones	Daidzein	H	H
	Genistein	H	OH
	Glycitein	OCH_3	H

Figure 4.2 Glucoside conjugated, and unconjugated (aglycone), structures of soy isoflavones

Following removal of the sugar, the aglycone may be metabolized further or absorbed to undergo first pass hepatic metabolism (Scalbert and Williamson, 2000, Xu *et al*., 1995, Kim *et al.,* 1998). These metabolic steps will significantly alter bioavailability (glycosides are poorly absorbed) and bioactivity (many of the microfloral and mammalian metabolites are pharmacologically inactive) (Hur *et al*., 2000, Xu *et al.,* 1995, Joannou *et al.,* 1995).

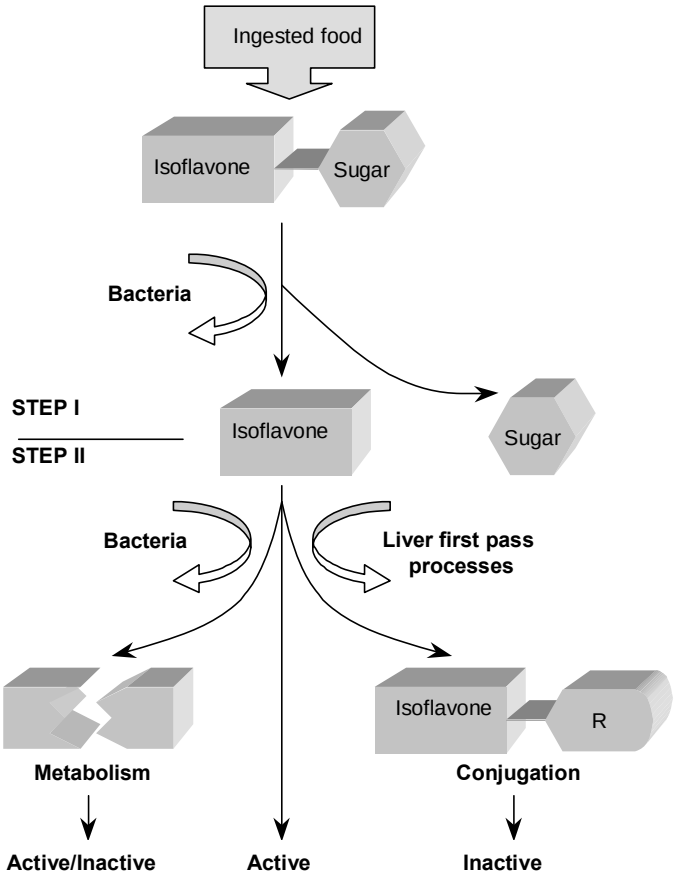


Figure 4.3: **Two-step process of isoflavone metabolism from food** (drawn by N.Turner).

Numerous experiments have demonstrated a significant rise in genistein and daidzein in the plasma after controlled feeding of isoflavones (usually as a glycoside-containing soy protein) to volunteers (Xu *et al*., 1995, Gooderham *et al.,* 1996, King and Bursill, 1998, Rowland *et al*., 2000, Setchell *et al*., 2001, Watanabe and Adlercreutz,1998, Xu *et al.,*1994). These support liberation of the aglycone prior to absorption. Glycosidases are important enzymes in the metabolic release of flavonoid aglycones. It is very well known that gut microflora produce glycosidases and are the most important source of these enzymes in the intestine (Hawkesworth *et al*., 1971, Rowland, 1986, Davenport, 1982). Hawksworth *et al*., (1971) reported the activities of enzymes produced by mammalian microflora. Of the five enzymes investigated, β-glucosidase and β-glucuronidase are most important in hydrolysis of glycosylated flavonoids. The β-glucuronidase is important when flavonoid glucuronides are excreted in bile during enterohepatic circulation

(Setchell *et al*., 2001*)*. Figure 4.4 shows the activities of each enzyme for the principal species of gut bacteria.

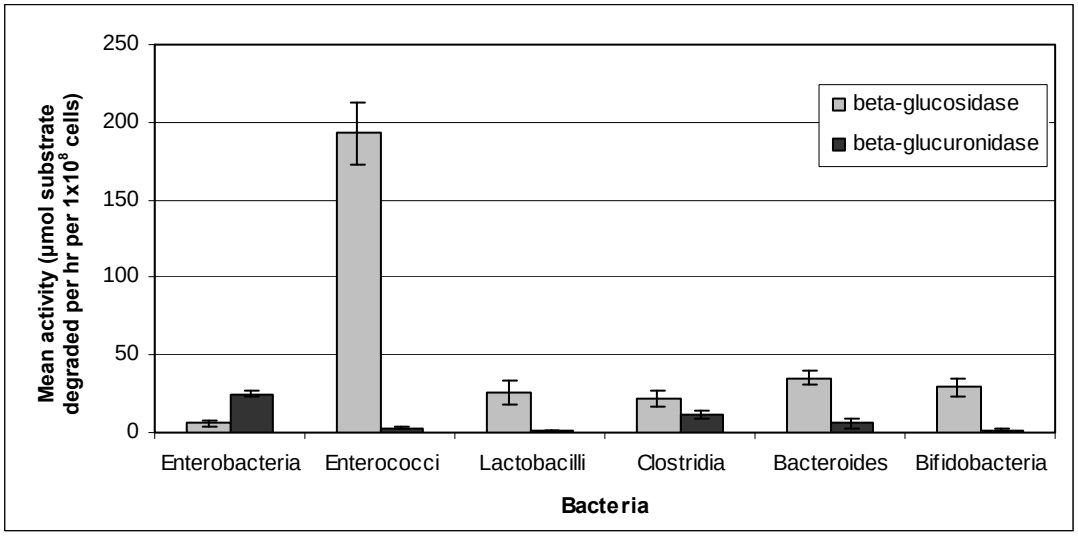


Figure 4.4: Mean glycosidase activities of the principle species of human gut bacteria (Hawkesworth *et al*., 1971)

Clearly, the enterococci (faecal streptococci) display the highest activity of β-glucosidase. Because these bacteria predominate in the small intestine, this has obvious implications for deglycosylation and uptake of aglycones in this region. The *Lactobacillus*, *Bacteroides,* and *Bifidobacterium* also have strong β-glucosidase activity. Enterobacteria, *Clostridium,* and *Bacteroides* have strong β-glucuronidase activity that is likely to be important in metabolism of enterohepatically recycled flavonoids (Kim *et al.,* 1998, Tannock, 2000). Four strains of *Bifidobacterium* demonstrated β-glucosidase by hydrolyzing isoflavones in soy milk (Tsangalis *et al*., 2002).

There is evidence that deglycosylation by gut microflora readily occurs. Indeed, genistein appears in plasma irrespective of whether it is consumed as the glycosylated form (genistin) or the aglycone (genistein). However, free isoflavones (e.g., genistein) reach peak plasma levels before the corresponding glycosylated form (e.g., genistin) (Setchell *et al.,* 2001). The presence of the sugar moiety therefore delays rather than inhibits bioavailability.

Following absorption of the aglycone, liver first pass phase II metabolism results in glucuronidation (Scalbert and Williamson, 2000). The resulting glucuronides are excreted in bile (Drasar and Barrow, 1985, Griffiths and Hackett, 1977), and gut microfloral β-glucuronidase may re-release the aglycone for reabsorption and further enterohepatic circulation, thus prolonging the pharmacologic activity of the isoflavones (Figure 4.5) (Drasar and Barrow, 1985).

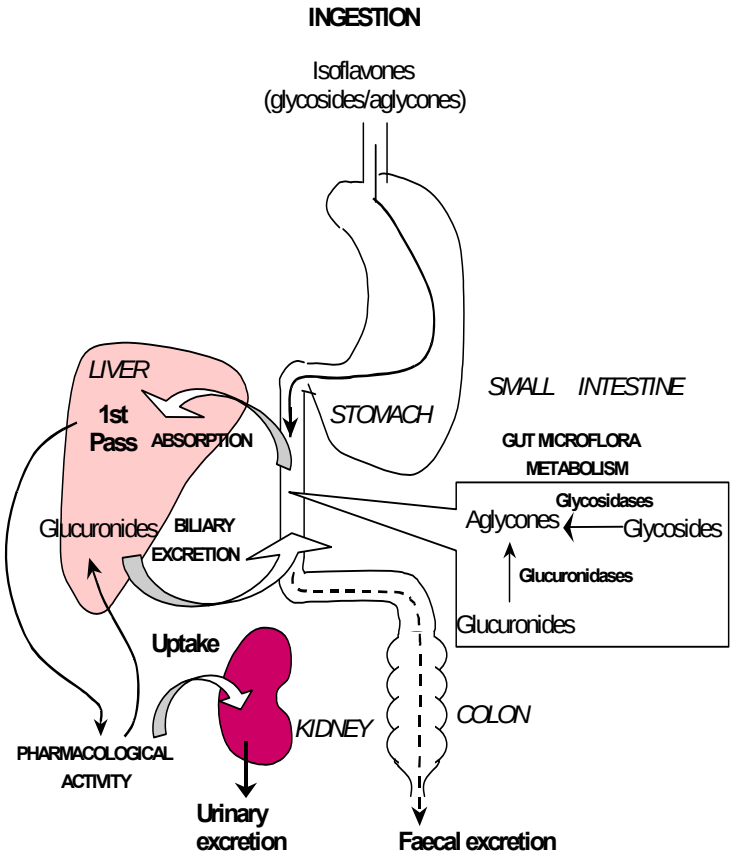


Figure 4.5: Schematic representation of the importance of gut microfloral metabolism on the pharmacologic activity of isoflavones (drawn by N.Turner).

4.1.3.2 Degradation and Bioavailability of the Aglycone

The gut microflora have an important role in the metabolism of isoflavone aglycones. Experiments involving administration of antibiotics to humans showed decreased excretion of bacterially produced phytoestrogen metabolites, and experiments with germ-free rats demonstrated a complete absence of excreted isoflavone metabolites (Rowland *et*

al., 1999). Infants on soy formula during the first 4 months of life (when gut bacteria are minimal) form only small amounts of the daidzein metabolite equol (Setchell *et al.,* 1997).

The metabolic products might be more or less bioactive than their parents. Studies on the microbial metabolism of isoflavones have shown that an important metabolite of genistein is the non-estrogenic *p*-ethyl phenol (Wiseman, 1999, Watanabe and Adlercreutz, 1998). This is an example of gut microfloral deactivation (Figure 4.2). The major daidzein metabolites are *o*-desmethylangolensin (*o*-DMA) and equol (7-hydroxyisoflavan) (Rowland *et al*., 2000). The latter is more estrogenic than daidzein, (Rowland *et al.,* 1999, Markiewicz *et al*., 1993) and is an example of gut microfloral metabolic activation (Figure 4.2). *o*-DMA is weakly estrogenic and may even be hormonally inert (Rowland *et al*., 1999, Joannou *et al*., 1995).

Only a small proportion (1–25%) of whole dietary phytoestrogens are excreted in urine (Wiseman *et al.,*2004). Therefore, they are either not absorbed from the gut, absorbed and released in bile followed by faecal excretion, or metabolized by gut microflora or in the liver (Scalbert and Williamson, 2000). The amounts of intact free aglycones in faeces are low (Watanabe and Adlercreutz, 1998); thus direct elimination via the faeces is not likely. Indeed, in a few studies Xu *et al*. showed total faecal excretion of isoflavones from their subjects was only 1 to 2% of the ingested amount (Xu *et al*., 1994, 1995). Therefore >75% of dietary isoflavones must be metabolized beyond deglycosylation. The gut microflora are likely to be important in this process, which would account for the low bioavailability of pharmacologically active isoflavones from food.

There have been several attempts to study the bioavailability of isoflavones from soy. Generally these studies have involved administration of a soy-based meal to human volunteers, followed by plasma, urine, and faecal sampling to determine isoflavones and their metabolites. Most studies discovered that there were inter-individual variations of isoflavone metabolism that might be attributed to gut microfloral differences (Kelly *et al.,* 1993, Setchell *et al.,* 1984, Lampe *et al.,* 1998, Rowland *et al*., 2000, Wiseman *et al*., 2004, Zheng *et al.,*2003,2004). The other, less significant factor affecting bioavailability is the food matrix (Gorbach and Goldin, 1992), the effect of which may be determined by gender (Lampe *et al*., 1998). The inter-individual differences in isoflavone metabolism

signify that a food such as soy may be considered functional for one person, but may not exert the same extent of functionality in another.

4.1.3.3 Region of absorption.

Little is known about where the phytoestrogens and their metabolites are absorbed along the intestine. There is a perception that isoflavone glycosides and the free aglycones are metabolized and absorbed in the colon because this is where there are a very great number of microflora (Scalbert and Williamson, 2000, Wiseman, 1999, King and Bursill, 1998, Setchell *et al*., 2001). However, this is unlikely, because very little absorption of anything but water, electrolytes, short chain fatty acids, vitamins, and some trace nutrients occurs in the colon (Spence and Mason, 1992, Davenport, 1982, Levitan and Wilson, 1974). Additionally, few enzymes, if any, are produced by the epithelial cells of the large intestine, and the absorptive villi structures are absent (Spence and Mason, 1992).

The physiology of the human digestive tract points to metabolism and absorption of isoflavones primarily taking place in the jejunum, which, along with the duodenum, is the principle area of absorption of hydrolyzed compounds (Anderson and Garner, 2000, Hawkesworth *et al*., 1971, Rowland, 1986). This is supported in human studies in which a short time (0.25–1.5 h) between dose and plasma detection of isoflavones was recorded (the time would be much longer if absorption occurred in the colon) (Xu *et al*, 1995, Rowland *et al*., 1999, King and Bursill, 1998, Setchell *et al*., 2001, Morton *et al*., 1997). One cannot discount the fact that the soy meals administered in these studies could have contained low amounts of unconjugated isoflavones, which may account for some of the early detection (Arai *et al*., 2000). The aglycones likely accounted for less than 5% of the total isoflavones (Wang and Murphy, 1994), however, which does not explain peak plasma levels being approached within the expected transit time along the small intestine (Xu *et al*., 1995, King and Bursill, 1998, Xu *et al*., 1994). Additionally, enterohepatic circulation in the small intestine (including the microflora-rich ileum) increases the residence time of the compounds and can be misleading by influencing the isoflavone plasma peak and half-life length (plasma peaks, which, by their timing, have been attributed to colon absorption are more likely the result of isoflavone plasma accumulation from recirculation).

4.1.3.4 Microflora involved

There is evidence that faecal bacteria extensively metabolize isoflavones (Xu *et al*, 1995, Hendrich *et al*., 1998, Zhang *et al*., 1999), and there have been attempts to identify the specific microbes that may be responsible. In one study a strain of *E coli* and an unidentified gram-positive bacteria were identified that could both convert daidzin and genistin glycosides to their respective aglycones (Hur *et al.,* 2000). The unidentified strain further metabolized the aglycones to dihydrodaidzein and dihydrogenistein, but a final metabolite for daidzein (equol) was not detected. There were other unidentified metabolically active bacteria in their experiments, but they were not pursued. Indeed, incubation of daidzein with a culture of crude faecal bacteria from which these species had been isolated resulted in the generation of equol, thus suggesting that several species could be involved at different stages of the metabolic pathway. A study using quercetin supported this hypothesis (Schnieder *et al*., 1999). More detailed work showed that strains of *Bacteroides*, *Eubacterium,* and *Fusobacterium* efficiently cleaved the sugar group from daidzein (Kim *et al*., 1998). Following this, phenolic acids were principally produced by another strain of *Bacteroides*. Ring fission is important in reduction of bioactivity of isoflavones, and was shown to be carried out by *Streptococcus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., and *Bacteroides* spp. All of these microflora inhabit the jejunum (Table 4.1). At this time, however, it is not obvious which microbe (or microbes) is principally responsible for the degradation of isoflavones in the small intestine.

4.2 Degradation of isoflavones by faecal microflora

There have been a number of reports where gut microflora have been exposed to isoflavones. Using faecal matter as a source of the total gut microbial population from one individual, Xu *et al*., (1995) traced the rate of genistein and daidzein degradation separately from an initial concentration of 120 μmol/l. Further work established average half-lives for genistein and daidzein (in combination, initial concentration 1180 μmol/L) of 8.9 h and 15.7 h respectively (n=14) and demonstrated differences between individuals (Zhang *et al*., 1999). Hendrich and co-workers (1998) reported relatively consistent rates of degradation of isoflavones by microflora from faecal samples of 15-20 subjects based

on measurements of activity at day 0 and at day 300. On the basis of this consistency, they suggested relatively stable human gut microfloral differences in the ability to degrade isoflavones. In further work, the same research group found subjects grouped into three phenotypes for daidzein and two phenotypes for genistein degradation based on the rate of disappearance of the isoflavones in faecal isoflavone incubations (Zheng *et al*., 2003). In addition subjects with a rapid gut transit time (GTT) and slow faecal degradation had higher urinary levels of genistein, indicating that gut transit time and faecal degradation rate impact on the bioavailability of genistein, although this was not evidenced for daidzein (Zheng *et al.,* 2004). Wiseman *et al.,* (2004) report 13 ± 12% and 30 ± 20% conversion of daidzein for consumers on a low (0.54 ± 0.58 mg total isoflavones/day) and high (104 ± 24 mg total isoflavones/day) soy diet respectively after 72 hours incubation.

To test the hypothesis that gut microflora activity accounts for the difference between estimated and measured serum levels from dietary intake of isoflavones a number of experiments were undertaken using aliquots of fresh human faecal samples incubated with genistein, daidzein or a mixture of the two isoflavones. Isoflavone concentrations, approximately 60 µmol/l for the individual isoflavones and 120 µmol/l for the isoflavones in combination, were measured, in the first instance by thin layer chromatography, and subsequently, by HPLC using methodology based on Xu *et al*., (1995). Details of the methodology are given in Chapter 8.4.

4.2.1 Cultural differences

The initial goal was to obtain preliminary information on genistein and daidzein degradation from individuals with different diets. Therefore, three cultural groups were targeted: Chinese (15-20 years) moved to New Zealand recently from China, New Zealand Maori and European New Zealanders. Informed consent and faecal samples were obtained from nine individuals comprising 6 European (1 vegetarian, 5 omnivores) and 3 Maori New Zealanders. Attempts to obtain samples from Chinese students were unsuccessful, ascribed to the sensitive nature of the sample in the Chinese culture. The assistance given and participation by all subjects is most gratefully acknowledged and respected.

An aliquot of homogenised, centrifuged faecal supernatant in growth broth was spiked with genistein (58µmol/l) and daidzein (39 µmol/l) and incubated at 37 °C (See Chapter 8.4 for details). 1ml aliquots were extracted and analysed by thin layer chromatography at time = 0, 5 and 24 hours and visualised under UV light (265nm) (Figure 4.6, Table 4.2). No difference was observed between the time 0 and 5 hour samples. After 24 hours, extracts from 6 of the 9 subjects showed decreased intensity of genistein with 4 of these subjects also showing a decrease in the daidzein intensity (but no evidence of the estrogenic metabolite, equol). Of the 6 'degraders' one was Maori (self claimed), one was a vegetarian, and 4 were European New Zealanders consuming a typical Western diet.

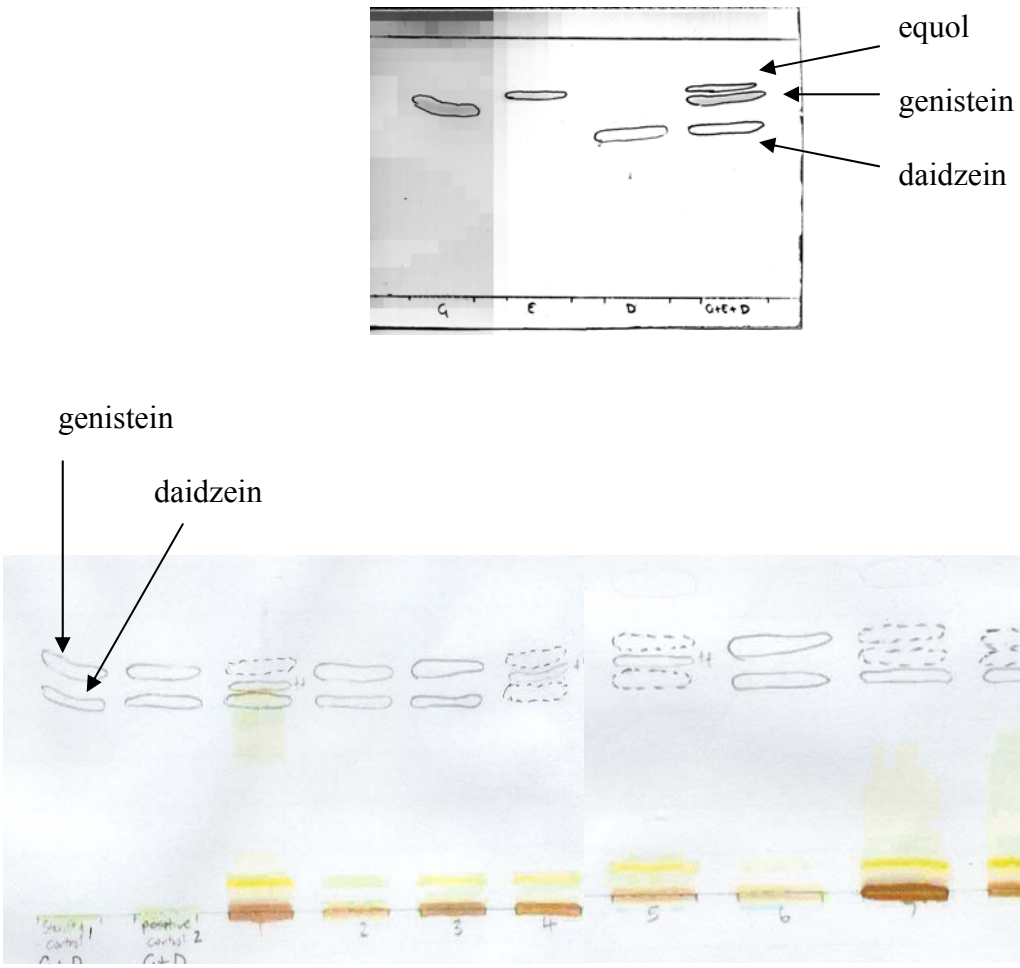


Figure 4.6: **Thin layer chromatographic results for standard solutions (genistein, equol , daidzein) above and of faecal extracts from 9 New Zealand subjects following 24 hours incubation with daidzein (39µmol/l) and genistein (58 µmol/l).** The duration of incubation supports the unidentified band being *p*-ethylphenol (a metabolite of genistein) rather than *O*-desmethylangolensin (a metabolite of daidzein).

Table 4.2: Thin layer chromatographic results of faecal extracts from 9 New Zealand subjects following 24 hours incubation with daidzein (39µmol/l) and genistein (58 µmol/l).

Extract		R_f	Intensity	Change from time 0	Identification
Sterility control (no bugs)		0.56	+++		daidzein
		0.64	+++		genistein
Positive control (E.Coli)		0.54	+++		daidzein
		0.62	+++		genistein
Subject 1	NZ European	0.54	+++	✗	daidzein
	(vegetarian)	0.64	++	✓	genistein
Subject 2	NZ Maori	0.54	+++	✗	daidzein
		0.62	+++	✗	genistein
Subject 3	NZ Maori	0.54	+++	✗	daidzein
		0.62	+++	✗	genistein
Subject 4	NZ Maori	0.54	++	✓	daidzein
		0.64	++	✓	genistein
Subject 5	NZ European	0.56	+	✓	daidzein
		0.65	+	✓	genistein
Subject 6	NZ European	0.54	+++	✗	daidzein
		0.64	+++	✗	genistein
Subject 7	NZ European	0.56	++	✓	daidzein
		0.67	+	✓	genistein
Subject 8	NZ European	0.56	++	✓	daidzein
		0.65	+	✓	genistein
Subject 9	NZ European	0.54	+++	✗	daidzein
		0.65	++	✓	genistein

Following these preliminary results, intra- and inter- individual differences in the ability to degrade isoflavones was quantified using high-pressure liquid chromatography (HPLC).

4.2.2 Quality control

Three control experiments were conducted to ensure validity of the extraction and detection methodologies. To assess reproducibility, some samples were set up in triplicate using faecal supernatant from Subject A in the three isoflavone conditions (each alone and mixed), and sampled over 72 h. Secondly, to assess efficiency of the Maxi-Clean cartridge extraction method, a solution of genistein and daidzein (59.8 and 64.5µmol/L respectively) was processed through prepared cartridges and the products collected at various points in the wash and elution process to be analysed by HPLC. Finally, the HPLC regime was calibrated against isoflavone standards.

The mean extraction recovery was 67% ± 12 (1 SD) and 64% ± 13 for genistein (n = 34) and daidzein (n = 31), respectively. Losses were not accounted for by the water rinses or 80% methanol elution and therefore most likely to be associated with lack of, or irreversible, absorption onto the cartridge. The HPLC response to the standards of genistein and daidzein was linear (R^2 =1.00) over the concentration range analysed (0-128 µmol/l).

4.2.2.1 Reproducibility

The reproducibility of degradative characteristics of triplicate faecal samples is shown in Figure 4.7. Whilst two of the replicates were similar, the third replicate was different with variability ascribed to a combination of analytical and microbial components. The negative degradation for daidzein (mixed with genistein) is indicative of analytical variability in the order of ±15% since the isoflavones cannot be synthesized by the microbes. The greater variability observed for 72 compared with 24 hours of incubation suggests a microbial component to the variability since more lifecycles give a greater chance for the microbial populations from 2 sub-samples to diverge. The limited reproducibility at longer incubation times means that differences observed in subsequent studies are preliminary only.

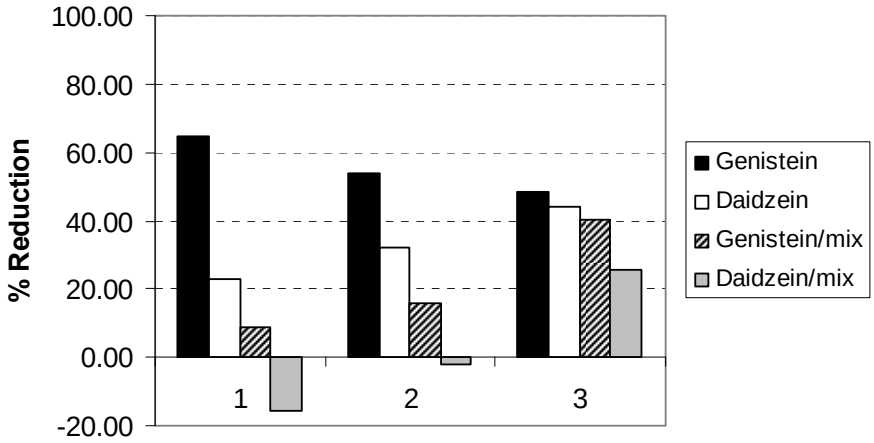


Figure 4.7: Reproducibility of degradation of isoflavones relative to time 0 for three replicates of a faecal sample (1,2,3) incubated up to 72 hours. Genistein 59.8, daidzein 64.5 and total mixed isoflavones at 124.3 µmol/l.

4.2.3 Intra-individual variation

In four out of five faecal samples taken over a 15 week period, the gut microflora in the faeces of Subject A showed degradation ability towards the isoflavones (Figure 4.8). In each case genistein and daidzein were added individually and together.

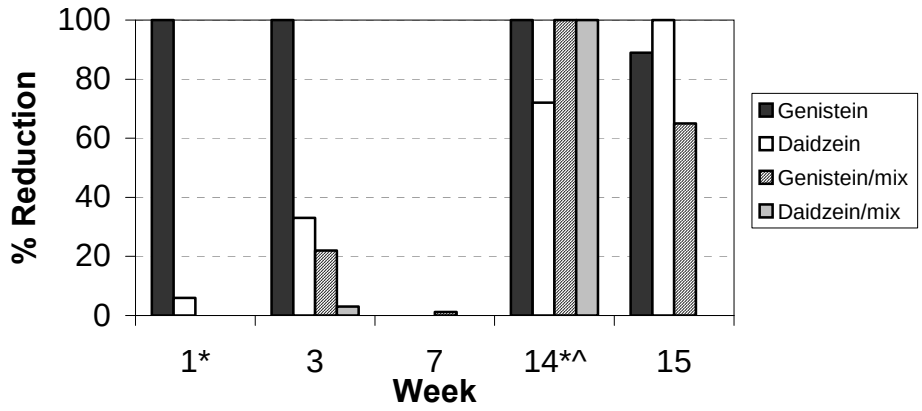


Figure 4.8: Total percentage change in concentration of isoflavones relative to time 0 h due to degradation by faecal microflora from Subject A in 5 experiments over 15 weeks. 'Mix' indicates where daidzein and genistein were present in combination. In all cases the initial concentration of isoflavone was 60 μmol/L (total 120 μmol/L when mixed), and experiments were concluded at 72 h. Exceptions are denoted with an (*) where the experiment was concluded at 28 h, and (^) where the starting concentration was 30 μmol/L (60 μmol/L mixed).

Little (within error) or no degradation of any isoflavone combination was observed at week 7, although samples from other subjects did show degradation and were therefore a positive control. Despite the experiment concluding within a shorter timeframe of incubation (28 h), much of the isoflavones were completely degraded in the week 14 sample, where initial concentrations equaled 30 μmol/L (total of 60 μmol/L in mixtures) – half the concentration of the other trials. For the remaining three trials, microflora from Subject A consistently degraded genistein alone within 30 h, but showed variable degradation ability for daidzein alone (30 and 90% where experiment allowed to run for 72 h, degradation had not occurred after 28 h). When present in an isoflavone mixture, little to no degradation of daidzein was seen, and degradation of genistein was up to 65% only where the experiment was allowed to run to 72 h.

4.2.4 Inter-individual variation

The results in Figure 4.9 show the change in isoflavone concentration relative to time 0 h for the 5 NZ European subjects. The experiment was concluded at 30 h (genistein), and 72 h for daidzein, genistein/mix (in combination with daidzein) and daidzein/mix (in combination with genistein), except where 100% degradation was detected beforehand.

The results demonstrate both the ability for each individual's faecal gut microflora to degrade isoflavones, and differences between individuals in the extent of degradation for faecal samples collected at one time point. Other than Subject A, who showed no isoflavone degradation ability in this trial, all subjects completely metabolised genistein within 24 h and daidzein to variable extents (100% detected at 36 h and 60 h for Subjects B and D respectively). In the mixtures, significant degradation of genistein was only observed in Subject C, and no daidzein metabolism was detected in the microflora of any participant.

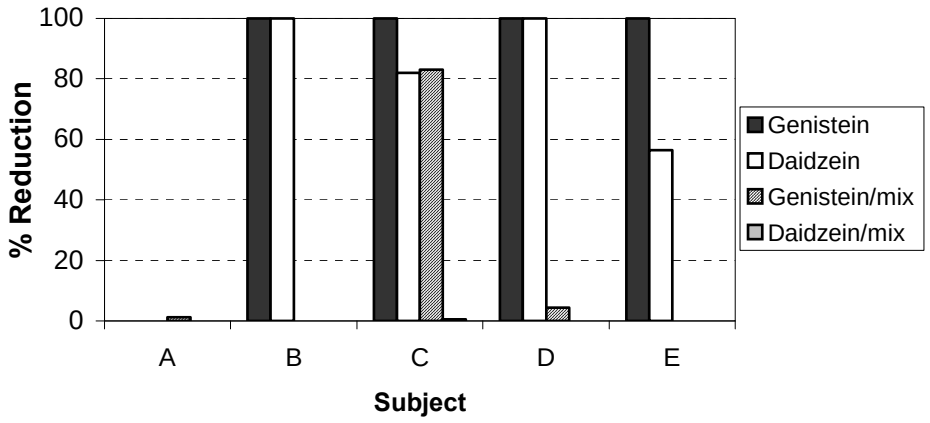


Figure 4.9: Percentage change in concentration of isoflavones relative to time 0 due to degradation by faecal microflora from 5 NZ European subjects. Genistein 59.8, daidzein 64.5 and total mixed isoflavones at 124.3 µmol/l.

4.2.5 Response at varying isoflavone concentration

The ability of the faecal microflora from 3 of the 4 subjects, to degrade both genistein and daidzein when added individually (at approx 60µmol/l), but not when presented as a mixture (at a total isoflavone concentration of 120 µmol/l), raised the question of whether

isoflavone degradation was concentration dependent. Figure 4.10 shows the maximum % degradation of the isoflavones (up to 72 hours) at varying isoflavone concentrations, for subject A. Isoflavones were added individually and in combination at varying isoflavone concentrations. Initial concentrations were 30, 60, 90, 120 and 150 µmol/l. For the mixtures, this represents the total starting concentration of the isoflavones combined (half of each of genistein and daidzein). There is a tailing off of degradation from a combined isoflavone concentration of 90µmol/l upwards, suggesting that higher isoflavone may be toxic to the microflora or that the microflora had reached maximum activity (had eaten enough isoflavone). Thus the reduction in isoflavones concentration at 60 µmol/l genistein and daidzein compared with at 120 µmol/l for 3 of the 4 subjects (Figure 4.8) was confirmed.

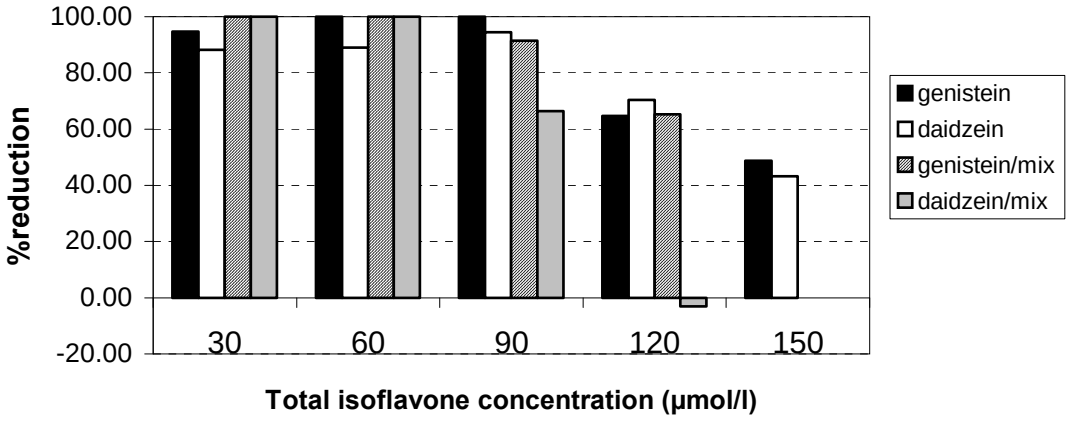


Figure 4.10: Percentage reduction of isoflavones at increasing isoflavone concentration in the microflora substrate, Subject A.

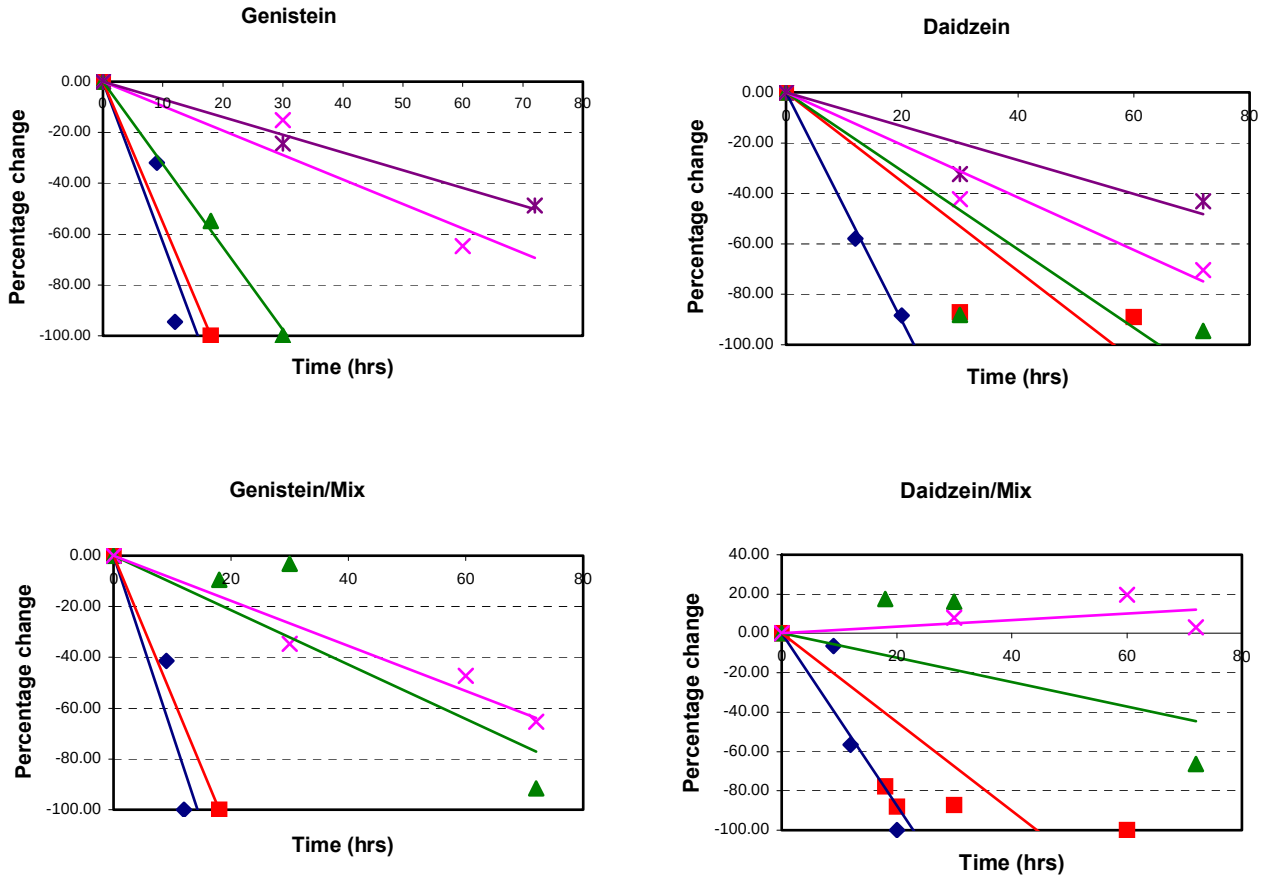


Figure 4.11: Effect of isoflavone concentration on rate of degradation Starting (time 0 h) concentrations of isoflavone : 30 (♦), 60 (■), 90 (▲), 120 (x) and 150 (✳) µmol/l.

The rate at which isoflavone concentration changed with varying substrate levels is shown in Figure 4.11. A linear trend-line was selected as the best representation across all results. The coefficient of determination (r-squared) ranged between 0.64-1.00 (excluding 2-point graphs) with a mean and median of 0.86. This indicated that the linear trend-line was a reasonable estimate of the true pattern. Clearly the rate of change in isoflavone concentration (implying degradation) decreased with increasing isoflavone concentration.

The positive trend line for daidzein, when present in a mixture at the highest total concentration (150 µmol/l), is a result of low isoflavone recovery and is considered indicative of nil degradation.

4.2.6 Effect of Pre-exposure

In one instance 4 ml faecal supernatant from Subject A was grown in the presence of genistein at a final concentration of 60 μmol/l in micro-aerophilic conditions at 37 ˚C (referred to as tube A). After 30 h, 1 ml was removed and inoculated into a fresh tube of growth medium and genistein to give a total of 5 ml at 60 μmol/l genistein (tube B). An appropriate amount of genistein was added to the original tube (A) to achieve a concentration of 60 μmol/l (C). The tubes were then re-incubated and samples analysed over the following 18 hours.

The time taken for complete degradation of genistein was approximately halved for a pre-exposed sample of faecal microflora (Figure 4.12).

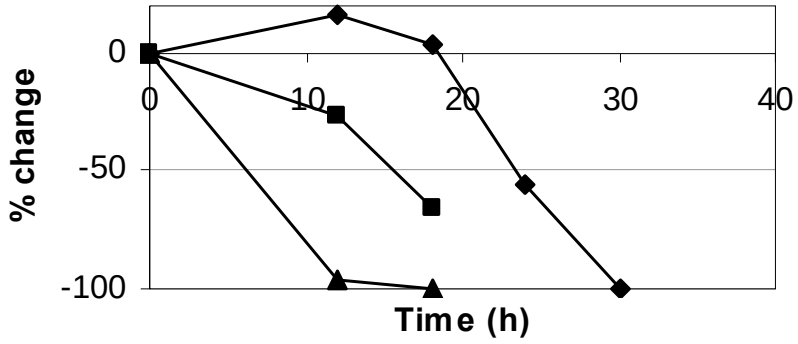


Figure 4.12: Impact of pre-exposure on the degradation rate of genistein by faecal microflora. (♦= original faecal culture (A), ■ = 1ml of original culture inoculated into fresh genistein/medium (B), ▲= original culture re-inoculated with genistein.

4.3 Discussion

The use of faecal material as a source of gut microflora is a pragmatic approach increasingly being used (Xu *et al*., 1995, Setchell *et al*., 1984, Zhang *et al*., 1999, Zheng *et al.,* 2003, 2004, Wiseman *et al.,* 2004, Simons *et al.,*2005). Ideally, microflora would be obtained from the small intestine where the majority of absorption is likely to occur. Relative proportions, rather than species diversity, of microflora change along the gastrointestinal tract (Table 4.1). Faecal material is therefore likely to oversample small

intestinal flora compared with the small intestine. The pH of the BHI broth used to incubate the microflora is at the upper end of the pH range for the small intestine (6-7.5, Boudinot, 2004) and it is possible that these studies may not represent the *in vivo* small intestine environment. The limited reproducibility achieved in these experiments means that results for incubations beyond 24 hours are preliminary only.

Subject A consumed a typical omnivorous diet with an estimated isoflavone intake of 1.0 mg/day genistein and 0.8 mg/day daidzein (Table 2.2.4b). Faecal incubations for this subject demonstrated considerable unpredictability in gut microfloral degradation ability when sampled 6 times over 3 months, despite no intentional soy consumption during this time. In 4 experiments where degradation was observed and quantified, genistein was metabolised to completion within 28 hours when present alone, but the rate and extent of metabolism of the daidzein and both isoflavones in mixture was variable. In one experiment, degradation of isoflavones (singularly or in combination) did not occur. This variability within an individual was also observed for one of the other European New Zealand subjects and is at variance with opinions of others (Hendrich *et al.,* 1998, Zheng *et al*. 2003, Wiseman *et al*., 2004). Zheng and co-workers (2003) found phenotypes were maintained when measured 10 months apart. Wiseman and colleagues (2004) reported similar plasma concentrations between mid-point and end-point samplings of a 10 week isoflavone feeding trial. It is appreciated that the results presented here are preliminary yet there is no evidence that the methodology failed as simultaneous incubations for other subjects showed degradation. No other investigators have reported more than duplicate studies. Further work involving more subjects is needed to confirm the overall pattern of metabolic ability with time in New Zealand Europeans, but the results, at least for Subject A, contradict the 'stable-microflora metabolism' conclusions.

A qualitative comparison of the isoflavone metabolic activity of the microflora from 9 New Zealanders comprising 3 Maori, and 6 European New Zealander (of which 1 was a vegetarian) showed that 6 subjects were 'degraders'. Quantitative studies with faecal samples from 5 of the European subjects showed remarkable differences in both absolute ability and rate of degradation. Comparing the degradation ability of genistein across all subjects, 4 out of 5 were able to completely degrade genistein within 24 h when present alone at a starting concentration of 60 μmol/l. Coupling this amount with 65 μmol/l daidzein, effectively doubling the total isoflavone concentration, basically eliminated all

degradation of genistein in 3 out of 4 of the genistein-degraders. A similar result was seen with daidzein, with 4 out of 5 subjects initially able to metabolise the compound to some extent when it was present alone, but none able to metabolise in mixture. This result suggested that there might be a concentration at which the isoflavones become inhibitory to the microflora, and that this point of inhibition was more dependent on the total concentration of isoflavones than the presence of either compound. Compromised metabolic activity of the microflora at high levels of isoflavone was supported by the absence of non-isoflavone metabolic products in the HPLC chromatograms, such as those from the growth medium.

Inter-individual differences in microbial degradation of isoflavones are consistent with the wide variation in plasma and urinary concentrations of isoflavones reported by other workers (Arai *et al*., 2000, Uehara *et al*., 2000, Wiseman *et al*.,2004). For example plasma levels for genistein and daidzein ranged from 5.0-2513 nmol/l and 1.7-861 nmol/l respectively for mean (± SD) intakes of 86.5 (± 47.0) and 57.4 (± 31.4) μmol/day (Uehara *et al.,* 2000). In the work undertaken by Wiseman and co-workers (2004), plasma levels of genistein and daidzein were highly variable 691 (± 690 nmol/l) and 369 ± 456 nmol/l respectively for 13 subjects who each consumed the same amount of isoflavones, namely 56 mg genistein and 43 mg daidzein.

Setchell *et al.,* (1984) reported the conversion of daidzein to equol by faecal microflora from studies of one individual. In controlled diet studies Rowland and co-workers (2000) found subjects fell into 2 categories, good (64% of subjects) and poor (36% of subjects) equol excretors, on the basis of urinary excretion. Based on relative retention times in the HPLC traces, equol was detected as a metabolite product in incubations from 2/5 of our subjects (40%). Microflora from two of our individuals were able to degrade daidzein with no evidence of equol production. Therefore the inter-individual variation in the rate and/or ability to degrade daidzein, by the microflora from our subjects, is not explained by the ability to produce equol.

The most likely reasons for gut bacteria to metabolise isoflavones are; the provision of nutrients for energy, their use in the construction of other compounds needed by the cell, because they are toxic to the cell or that they are co-metabolised in the process of another reaction. On the basis of the results obtained it was hypothesised that as the total isoflavone concentration approached 120 μmol/l, the rate of degradation would decrease

and no degradation would be seen at this concentration. However, in the experiments designed to show this, degradation of at least 40% was observed in all isoflavone combinations except for the daidzein portion of the 120 μmol/L mixed samples. Though the data are limited, a picture emerges (Figure 4.8) that suggests the rate of degradation decreases, and hence bioavailability increases, at increasing isoflavone concentration. However, this is not supported by % transfer of genistein and daidzein to urine at different intakes (Table 4.3) and requires confirmation.

Table 4.3: Recovery of isoflavones in urine as a percentage of intake

Isoflavone	Intake (μmol/day)	% recovery in urine
Genistein	206[1]	8
	129[2]	7
	112[3]	18
Daidzein	170[1]	15
	84[2]	21
	103[3]	36

1 = Wiseman *et al.,* 2004, 2=Rowland *et al.,* 2000, 3=Watanabe *et al.,* 1998.

With the majority of digestion and absorption occurring in the proximal small intestine, microbial activities with significance to bioavailability of isoflavones are most relevant within the 3-6 h period it takes for food to pass through this region (Spence and Mason, 1992). Results from the studies described in this chapter, showed a delay of around 18 hour before degradation was significantly detected, suggestive of an incubation lag period necessary for an *in vitro* minority population of bacteria to grow to high levels before activity becomes recognizeable. This initial delay was much reduced (50% degraded in 6h or less) when faecal microflora had been pre-exposed to genistein. While acknowledging likely *in vitro* selection factors in this experiment, it is speculated that in the natural gut habitat microflora have the ability to metabolise genistein within the time needed for food to pass through the upper small intestine, where absorption is at its peak (Spence and Mason, 1992). This is because the gut acts as a continuous culture with continuous exposure to estrogenic compounds. On the other hand, it appears unlikely that daidzein will be degraded in significant amounts within this period due to its much slower (and sometimes absent) metabolism. This suggests that daidzein is more bioavailable, consistent with the higher proportions of daidzein found in urine compared with genistein (Table 4.3).

4.3 Summary

Maximal absorption of bioactive food components is likely to be in the proximal small intestine (duodenum, jejunum). This region of the gastrointestinal tract harbors microflora dominated by lactic acid bacteria, *Streptococcus*, *Lactobacillus,* and *Bifidobacterium*, with the latter group only present if anaerobicity is sufficient. Other human-associated genera are also present in varying population sizes. Thus there are a wide range of bacteria that may potentially be involved in the degradation and bioavailability of isoflavones. The identity of the species involved is not well understood.

Intestinal bacteria express several glycosidase enzymes, with *Enterococcus* (*Streptococcus*) demonstrating particularly high amounts of β-glucosidase activity, followed by *Lactobacillus*, *Bacteroides,* and *Bifidobacterium*. Clearly, small intestinal microflora have ample capacity to hydrolyze any glycosidated isoflavones ingested by the host, releasing the bioactive aglycone for absorption or further metabolism. Enterobacteria, *Clostridium,* and *Bacteroides*, all significantly present in the ileum, show strong β-glucuronidase expression. These organisms are important in hydrolyzing inactive aglycone glucuronides released in the bile via enterohepatic circulation, similarly making the aglycone bioavailable to host and microbes alike.

The role of gut microflora in the bioavailability of the active constituents genistein and daidzein is highly variable between individuals, and possibly within an individual and at least at the present time, unpredictable. These findings have important implications for the promotion and prescription of soy foods and supplements for disease prevention and health benefits (such as an alternative to HRT) since the bioavailable dose is highly variable.

The low rate of degradation of daidzein by gut microflora found in this study and elsewhere (Wiseman *et al*., 2004) coupled with a transit time of 3-6 hours through the upper intestine, mean it is difficult to accept degradation of the aglycone form by gut microflora as an explanation for the difference between estimated and measured plasma levels from dietary intake of isoflavones (Chapter 2).

Chapter 5

The establishment and validation of the yeast assay for estrogenicity

5.1 Introduction

The "yeast" assay is a "gene expression" assay for measuring the estrogenicity of compounds that can interact with the human estrogen receptor (hER). Yeast cells do not normally contain an estrogen receptor, however the DNA sequence of the human ERα has been stably integrated into the main chromosome of yeast (*Saccharomyces cerevisiae*) by the Genetics Department at Glaxo. In 1996, Routledge and Sumpter published methodology for an assay based on this modified yeast (Routledge and Sumpter, 1996). The schematics of this assay are shown in Figure 5.1

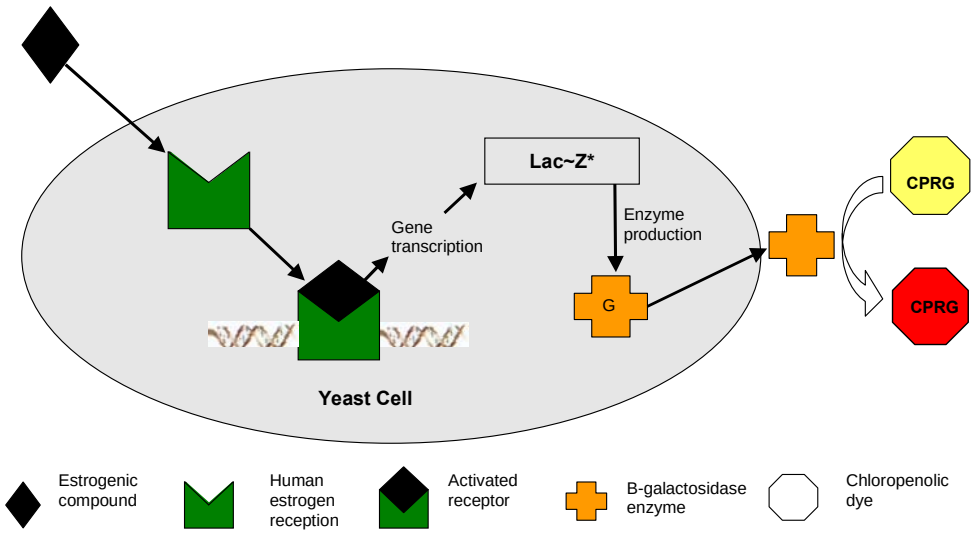


Figure 5.1: Schematic for the basis of the yeast assay using yeast transfected with the human estrogen receptor (ERα).

Yeast cells contain expression plasmids for the reporter gene *lac-Z.* This gene codes for the expression of β-galactosidase enzyme, that cleaves a dye added to the assay medium. The binding of a compound to the estrogen receptor results in gene transcription, that causes expression of the reporter gene *lac-Z,* and production of the enzyme β-galactosidase. The enzyme is secreted into the medium, where it metabolizes the chromogenic dye, chlorophenol red-β-D-galactopyranoside (CPRG), which is normally yellow, into a red product. The absorbance of the red colour is measured at 540nm. Estrogenicity is quantified relative to the absorbance obtained from the endogenous hormone 17β-estradiol.

The assay has been used to measure the estrogenicity of a wide range of compounds including: surfactants and their primary degradation products, the synthetic plasticiser bisphenol A, insecticides, herbicides, flavonoids and isoflavones (Arnold *et al*., 1996, Breinholt and Larsen, 1998, Connor *et al*., 1996, De Boever *et al*., 2001, Routledge and Sumpter, 1996). Environmental applications have included the estrogenicity of compounds in water and wastewater (Murk *et al*., 2002; Garcia-Reyero *et al*., 2001) and sediments (Legler *et al*., 2002).

In this work, the yeast assay was established and validated, in conjunction with fellow PhD student, Anna McCarthy (Chemistry Dept., University of Canterbury) with the aim of using it to measure the transfer of estrogenic compounds across the human placenta (Chapter 6).

5.2 Establishment of assay

5.2.1 Importation of the yeast

Since the yeast is genetically modified, importation required that New Zealand biosecurity regulations were met. Communications with ERMA (Environmental Risk Management Agency) were pursued until authority was gained to import the strain under an ERMA approval code NOC000705, for NOC99011, covering the importation into containment, for research purposes, genetically modified *Saccharomyces cerevisiae* laboratory strains

that contain fragments of DNA cloned from other species. The yeast were imported into ESR's containment facility under MAF permit #2002015009.
On arrival, the 5 yeast slopes were grown at 32º for 3 days whence 1ml 30% sterile glycerol was added and aliquots taken for storage at –80º C.

5.2.2 Establishment of the assay

Assays were undertaken using standard solutions and following the protocols in Chapter 8.5. A total of 12 assay runs were undertaken before reproducibility and appropriate concentration ranges for the standards of interest were achieved. The reason for the variability and unpredictability of the results was found to be largely due to an excess of yeast cells being added to the growth medium. In our hands, the assay uses less than the amount specified with the assay instructions (250µl rather than 0.5-2.0 ml). With a chemist's approach, measured volumes of 24 hr yeast culture were added to the assay medium rather than a volume based on cell count.

The growth of the yeast culture was measured over a period of 24 hours, to determine the growth phase of the yeast. A cell count was also performed to verify that the amount of cells in the 24h culture. From Figure 5.2 it is seen that at 24 hours the cells are in a stationary phase of growth.

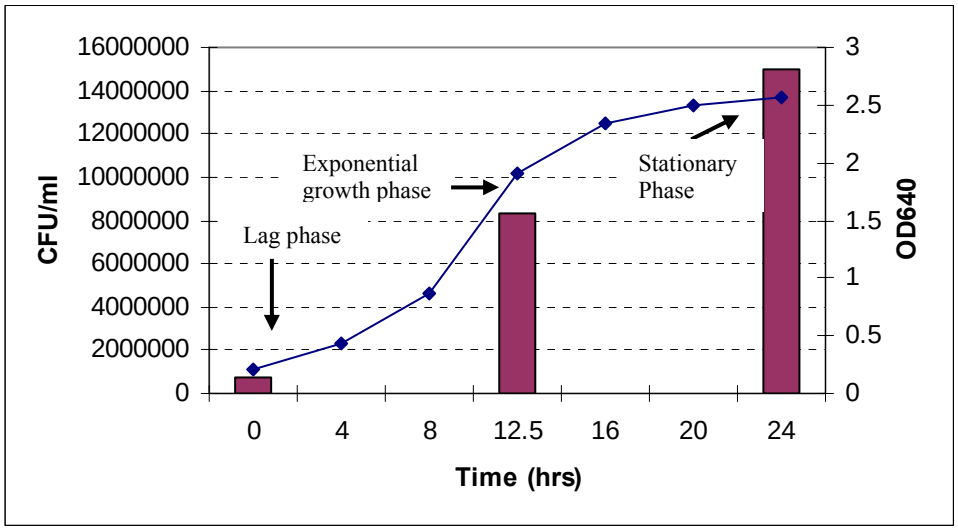


Figure 5.2: Growth of hER yeast over 24 hours measured by cell count (CFU/ml) and absorbance at 640nm

Since the estrogenic potency is different for different compounds, varying concentrations of the starting solutions were required to give a spread of absorbance readings across the serial dilutions of the test compounds. Standard solutions of 17β-estradiol (54.48 µg/l), genistein (278 mg/l) and bisphenol A (349 mg/l) resulted in a gradation of colour change across the plate (Figure 5.3).

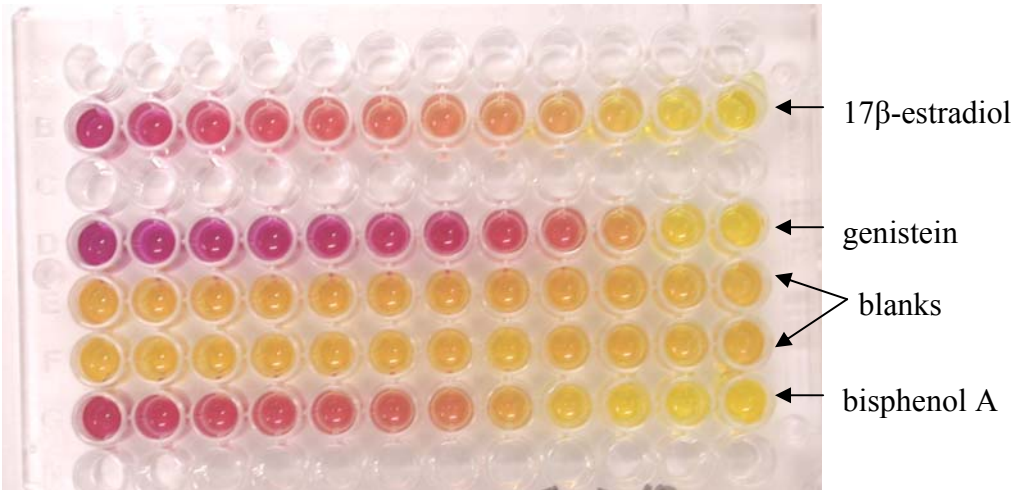


Figure 5.2: Colour changes observed from the yeast assay for starting concentrations of 17β-estradiol (54.48 µg/l: 10-0.005nM across the plate), genistein (278 mg/l: 51-0.03µM across the plate) and bisphenol A (349 mg/l:76-0.04µM across the plate)

5.3 Validation

Replicate 'S' curves were obtained for standard solutions of 17β-estradiol (n=8), genistein (n=6) and bisphenol A (n=4) Figures 5.4a and 5.4b. A single standard curve was achieved for nonylphenol but because this compound creeps on the well plate, with the risk of contaminating other samples, replicates were not pursued. Data points were fitted to a computer generated line fit using the software package 'SigmaPlot". From this line, EC_{50} values were determined where EC_{50} is the concentration of compound required to induce 50% of maximum estrogenic effect.

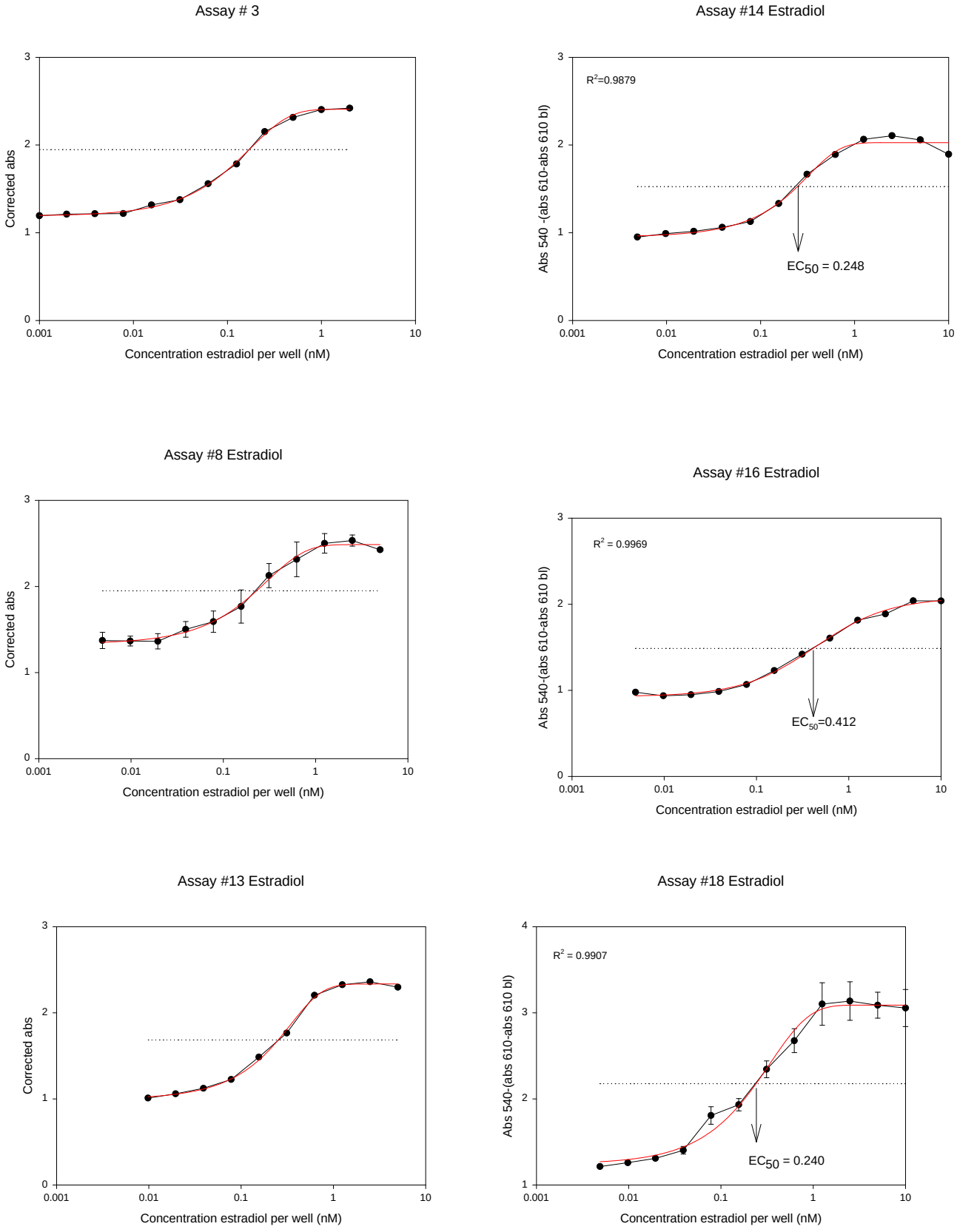


Figure 5.4a Standard curves for estradiol for six different assay experiments

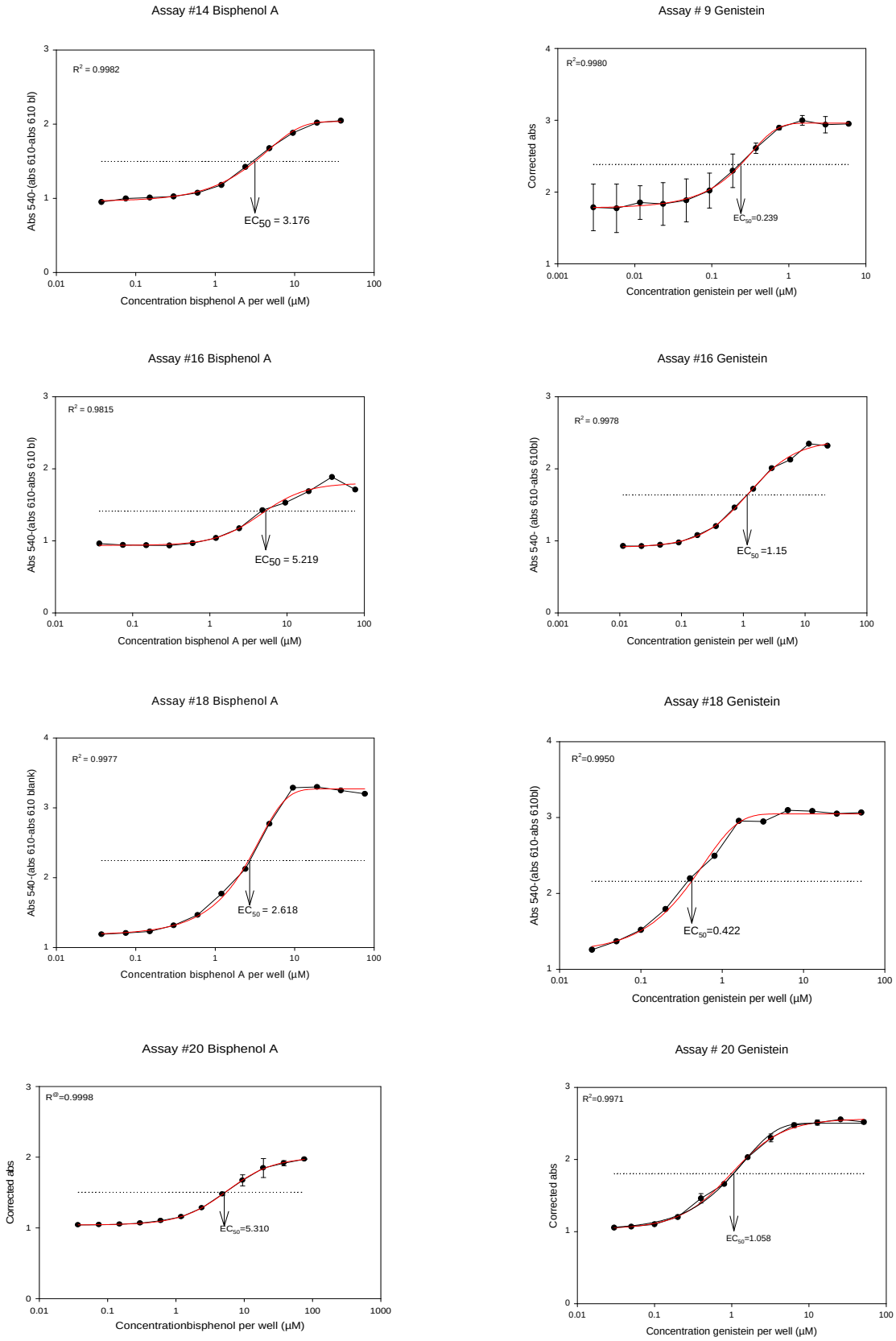


Figure 5.4b: Standard curves for bisphenol A and genistein from four different experiments

The assay is based on a biological system and whilst absolute values vary from one assay to another, depending for example on the number of yeast cells to begin with, the relative responses to different chemicals are comparable. The EC_{50} is the concentration of compound that gives half the maximum estrogenicity for that compound. By taking a concentration value at a common point on the standard curve, it is possible to measure relative estrogenicity of one compound to another. The variability of EC_{50} values (derived from SigmaPlot) and relative estrogenicities of estradiol, genistein and bisphenol A, as measured by the yeast assay, are shown in Table 5.1.

Table 5.1: Reproducibility of estrogenicity and relative estrogenicity for estradiol, genistein and bisphenol A using the yeast assay (EC_{50} per well= nM)

Assay #	EC $_{50}$ estradiol	EC $_{50}$ bisphenol A	EC $_{50}$ bisphenol A / EC $_{50}$ estradiol	EC $_{50}$ genistein	EC $_{50}$ genistein / EC $_{50}$ estradiol
3	0.125				
7	0.261				
8	0.228			519	2276
9				239	
13	0.252			489	1940
14	0.248	3176	12806		
16	0.412	5219	12667	1150	2791
18	0.240	2618	10908	422	1758
20	0.396	5310	13409	1058	2672
mean	**0.270**	**4081**	**12448**	**646**	**2192**
STD	0.093	1386	1076	369	448
STD (% of mean)	34	34	9	57	20

Whilst the variability of one standard deviation from the mean EC $_{50}$ ranges from 34 to 57 % between assays, the relative estrogenicity of genistein and bisphenol A to estradiol is less variable (9 and 20%).

A comparison between the EC_{50} for 17β-estradiol from this work and published results is shown in Table 5.2. There is good agreement between this work and results obtained by Tyler *et al.*,(2000), Beresford *et al*., (2000) and De Boever *et al.*, (2001).

Table 5.2: Comparison of EC_{50} values for 17β-estradiol

EC_{50} 17β-estradiol	Reference
$2.7 \pm 0.93 \times 10^{-10}$ M	This work
$2.1 \pm 0.35 \times 10^{-10}$ M	Tyler *et al*., 2000
$2.2 \pm 0.22 \times 10^{-10}$ M	Beresford *et al*., 2000
$1.44\text{-}10.78 \times 10^{-10}$ M	De Boever *et al*., 2001
$2.3 \pm 0.41 \times 10^{-11}$ M	Breinholt and Larsen, 1998

5.4 Limit of detection

Because the estrogenic potency of the xenoestrogens genistein and bisphenol A is in the order of 10,000 times lower than for 17β- estradiol when measured by the yeast assay, concentrations of starting solutions of genistein and bisphenol A have to be in the order of 10,000 times higher than17β- estradiol to detect a measureable colour change in the assay. This means that test solutions need to be at a concentration of approximately 300 mg/l for estrogenicity to be detected. Whilst this is possible for standard solutions, it will be difficult to obtain extracts of environmental or food samples that are sufficiently concentrated. This is likely to limit the usefulness of the assay for environmental samples. The assay, is however, a useful method for comparing estrogenic potency of pure compounds.

5.5 Summary

The yeast assay employs a yeast strain that includes the human estrogen receptor. Binding of an estrogenic compound to the receptor results in the activation of a gene that encodes for an enzyme that can cause a measureable colour change of a dye. Thus the assay is based on "gene expression". The assay has a reproducibility of 10-20% for potencies measured relative to a standard compound. The assay is useful for test compounds to be prepared at a starting concentration in the order of 300 mg/l.

Chapter 6

Xenoestrogen exposure during pregnancy -use of a placental model to study *in utero* exposure

6.1 Introduction

6.1.1 The DES Story

DES (Figure 1.1), is a potent estrogenic compound synthesized by Dodds and colleagues in 1938, that was prescribed to women in America and Europe to prevent miscarriage and other pregnancy complications (Toppari *et al.*,1996, CDC, 2004). Between 1938 and 1971 as many as 4 million women in the United States and 400,000 women in Europe were given DES. Although studies by Dieckmann and co-workers in 1953 demonstrated that DES was not effective for either of these therapies (Dieckmann *et* al., 1953) it continued to be prescribed (CDC, 2004). Then in 1971 Herbst and colleagues published a scientific paper in The Lancet that linked the occurrence of rare cervical and vaginal cancers in a small group of young women he was treating to the use of DES by the mothers of these patients (Herbst *et al*., 1971). This finding lead to a warning from the U.S. Food and Drug Administration about potential harmful effects of prescribing DES to pregnant women. The paper by Herbst *et al*. (1971) was pivotal in that it was the first example of transplacental carcinogenesis in humans. In other words, a chemical is transferred from the maternal circulation, across the placenta, into the fetal circulation and causes a change resulting in cancer some years after birth. In the case of DES and vaginal cancer, this was usually 15 to 30 years after exposure *in utero* (McLachlan *et al.*, 2001).

Although the consequences of DES exposure *in utero* seem less severe for boys than girls, some concerns have been raised. The sons of women who took DES during pregnancy are three times more likely to have genital structural abnormalities than men without such exposure (Cosgrove *et al.,* 1977). Men with *in utero* exposure also have sperm and semen abnormalities (but without an increased risk of infertility or sexual dysfunction) (Wilcox *et* al., 1995). Although individual studies of a link between maternal exposure to DES and an increased incidence of testicular cancer are variable (Henderson *et al*., 1988), a meta analysis of studies suggested a 2-fold increased risk of testicular cancer in the sons of mothers prescribed DES (Toppari *et al*., 1996).

The DES story continues with possible third generational effects. Alerted by two case reports, Klip *et al*., (2002) undertook a cohort study of 16,284 Dutch women with a diagnosis of fertility problems. The prevalence ratio of hypospadias in the 205 sons of women who were exposed to DES *in utero* compared with the remaining 8729 sons was 21:3. Although the absolute risk is small (2% of exposed mothers of boys), the evidence of placental transfer of an estrogenic compound affecting not just one, but two, subsequent generations is novel, significant and of concern.

Whilst recognizing that DES is not a dietary source of xenoestrogens, the work with DES is important because it provides evidence of placental transfer of a xenoestrogen from mother to fetus.

6.1.2 Evidence of *in utero* effects from environmental chemicals

Some pesticides used in agriculture, including DDT, dieldrin, endosulphan and synthetic pyrethroids have been shown to be estrogenic (Go *et al.,* 1999, Jorgensen *et al.,* 2000*,* Soto *et al*., 1995). Weidner and colleagues (1998) reported a significantly increased risk of cryptorchidism, but not hypospadias in the sons of women working in gardening. The subjects were Danish, the adjusted odds ratio= 1.67; 95% confidence interval 1.14-2.47 (Weidner *et al*., 1998). The risks were not increased in sons of men working in farming or gardening. The implication is that *in utero* exposure to agricultural chemicals resulted in the small increased risk of cryptorchidism. In a more recent study, maternal exposure to environmental pollutants at levels currently encountered in New York City have been associated with reduced fetal development (lower birth weights, body lengths and head

circumference) in the babies of exposed mothers (Perera *et al*., 2003). Swan and colleagues (2005) found an association between the concentration of four phthalate metabolites in maternal urine and anogenital distance (AGI), penis size and incomplete testicular descent in the sons of these women. A comparison of boys with prenatal phthalate concentrations in the highest quartile to those in the lowest quartile showed odds ratios of 10.2, 4.7, 3.8 and 7.3. The median concentrations of phthalate metabolites associated with the shorter AGI and incomplete testicular descent were within the range found for American women, based on nation-wide sampling. Phthalates are anti-androgenic, potentially interfering with the production of testosterone, rather than estrogenic but this work provides strong evidence of a link between prenatal exposure to an EDC and human male reproductive development.

6.1.3 Evidence for placental transfer of genistein and bisphenol A

The results reported by Herbst *et al*. (1971) have stimulated three decades of research into the understanding of hormones, our chemical environment and embryonic development (McLachlan *et al*. 2001). Included in this body of work are the following animal studies of placental transfer of the xenoestrogens genistein and BPA after maternal exposure. Genistein and BPA were selected as xenoestrogens of particular interest because of their contribution to estrogenicity from the diet (Chapter 2, Figures 2.1 and 2.13) and because they represent naturally occurring and synthetic compounds respectively.

6.1.3.1 Bisphenol A

In 1999 Miyakoda and co-workers found that BPA orally administered at a dose of 10 mg/kg transfers from maternal rat to the fetus (Miyakoda *et al*., 1999). The concentration of BPA in maternal blood plasma and fetal organs peaked at approximately 34 and 11 μg/l respectively within 1 hour of administration. Placental transfer of BPA has also been reported following a single subcutaneous dose of BPA administered to pregnant mice and monkeys (Uchida *et al.,* 2002). These results suggest that BPA passes easily through the placental barrier in a number of species.

Limited reports from animal studies of the effect of *in utero* exposure to BPA give mixed results. Prenatal exposure to a dose of bisphenol A comparable to levels found in the environment was found to lead to increased growth and early sexual maturity in female

mice (Howdeshell *et al*., 1999). On the other hand, Yoshino and co-workers (2002) concluded that exposure of female rats to BPA at concentrations up to 120 mg/kg/day during pregnancy and lactation does not result in any morphological abnormalities in the accessory sex organs (testis, epididymis, prostate and seminal vesicles) or spermatogenesis of male offspring. This means that even if BPA is crossing the placental barrier, it is not leading to abnormalities in the male rat reproductive outcomes examined.

Naciff and co-workers (2002) found that transplacental exposure of rats to the synthetic estrogen, 17 α-ethynyl estradiol, the natural phytoestrogen, genistein, and to bisphenol A, changes the gene expression profile of uterus and ovarian tissue in the fetuses of the exposed mothers, evidence that these estrogenic compounds cross the placenta in rats and have a biochemical effect.

6.1.3.2 Genistein

There is surprisingly little information available on placental transfer of genistein.

High levels of phytoestrogens, including genistein, in chord and amniotic fluid samples of healthy Japanese infants relative to maternal plasma levels indicated ready placental transfer of genistein and other phytoestrogens (Adlercreutz *et al.,* 1999*).*

Doerge and colleagues (2001) measured the concentration of genistein (conjugated and unconjugated) in maternal and offspring serum and brain following oral administration of genistein (20, 34 and 75 mg/kg bw) to pregnant female rats. Genistein concentrations were lower in fetal serum compared to maternal serum and unlike maternal serum, concentrations did not increase dose dependently. They found that a greater proportion of genistein was present in fetal serum in the unconjugated form. The concentration of the unconjugated genistein in the fetal serum ranged for 7-30% of the concentration in the maternal serum, showing that up to one third of the bioactive form of genistein crosses the rat placenta.

A warning "Don't eat soya if you're pregnant" was printed in NewScientist, 15 February 2003 (Jones, 2003). This article featured a study reporting the development of sex organs and sexual behaviour of rats exposed to genistein-supplemented diets during early

development (Wisniewski *et al* 2003). To determine if exposure to genistein at gestation and lactation at doses common in human diets, alters masculinization, the scientists examined the development of external genitalia, testes, wolffian ducts and sexual behaviour in male rats exposed to genistein-supplemented diets during early development. Exposure to genistein resulted in temporary, prepubertal morphological abnormalities and long-term dysfunction in reproductive behaviour, in which adult males exposed to genistein were less likely to mount and ejaculate. Also, males exposed to genistein had lower testosterone concentrations in adulthood. This work also demonstrated placental transfer of genistein in rats.

6.1.4 Sexual differentiation

An early mammalian embryo has the potential to develop either male or female reproductive tracts with sexual differentiation in humans occurring at 5-8 weeks of gestation. At this time the SRY gene on the Y chromosome produces an enzyme that stimulates development of the testis. SRY is the acronym for Sex-determining Region of the Y chromosome. In the absence of this enzyme ovaries develop. Once testes have formed they secrete a peptide (anti-Mullerian hormone), which causes regression of the potential female reproductive tract, and formation of testosterone. Testosterone in turn stimulates development of the male reproductive tract (Tortora and Grabowski, 2003).

Things can go wrong. If a genotypic male fails to make testosterone, it will not masculinize and will develop as a phenotypic female (but with testes). Conversely, if a genotypic female is exposed to sufficient testosterone she will be masculinized (but will have ovaries). The effect may be partial. Other organ systems such as the brain, liver and muscles are also "imprinted" during development and hence may be targets for xenoestrogens that perturb the normal endocrine profile at various stages of life. The greatest concern about environmental hormone disruptors arises from the possibility of irreversible effects of short term exposure at a critical period of life (WHO 2002a, 2002).

A number of genes are involved in the process of sex determination and differentiation and in a recent review article MacLaughlin and Donahoe (2004) discuss how mutations in these genes can lead to sexual abnormalities such as male pseudohermaphroditism. Since these are the types of effects observed in wildlife after exposure to xenoestrogens, it

seems highly conceivable to the author that compounds that alter expression of these genes at the critical period of differentiation could lead to sexual abnormalities.

6.1.5 Development of endocrine organs

Different parts of the endocrine organs develop at the following times:- pituitary (3 weeks), thyroid (4 weeks), pancreas and thymus (5 weeks) and pineal (7 weeks) (Tortora and Grabowski, 2003). Homeostasis of the endocrine system is established during fetal development and an abnormal environment, such as exposure to xenoestrogens, at this stage of life can result in permanent misprogramming (WHO, 2002a, COT, 2003).

6.1.6 Circulating levels of estradiol

The normal circulating levels of estradiol for a female are shown in Table 6.1. Estradiol originates from the maternal ovaries in the non-pregnant female and for the for the first 5-6 weeks of gestation, after which time production of estradiol switches to the placenta (Tortora and Grabowski, 2003). The serum concentration of estradiol varies from about 0.020 to 0.350 µg/l during the menstrual cycle.

Table 6.1: Normal circulating levels of estradiol for a female (Greespan and Gardner, 2001, Beard and Nathanielsz, 1984)

Time period	Serum concentration	
	µg/l	nM
1-5 years	0.005-0.010	0.018-0.037
6-9 years	0.005-0.060	0.018-0.220
10-11 years	0.005-0.300	0.018-1.100
14-16 years	0.020-0.068	0.073-0.250
Early follicular	0.020-0.100	0.073-0.357
Preovulatory and luteal	0.100-0.350	0.367-1.285
Post menopausal	0.010-0.030	0.037-0.110
Pregnant	0.100-30	0.367-110

In the pregnant female, estradiol rises to about 30 µg/l as shown in Table 6.2. At the time of sexual differentiation (5-8 weeks), which is reasonably a critical time for exposure

effects from environmental hormones the circulating level of estradiol in maternal serum increases from approximately 0.45 to 4.0 µg/l (Beard and Nathanielsz, 1984).

Table 6.2: Changes in serum concentration of estradiol during pregnancy (modified from Beard and Nathanielsz, 1984)

Gestational age (weeks)	Serum concentration (µg/l)
1	0.05
5	0.45
8	4.0
16	5.0
24	10.0
40	30.0

6.1.7 The Placental Model

Dual perfusion (i.e. separate maternal and fetal circulations) of a human placenta *ex vivo* is a technique whereby a placenta is obtained from a consenting mother immediately after delivery and solutions (perfusates) that maintain the placental tissue are circulated through a fetal lobe and a maternal lobe of the placenta. A schematic of the model is shown in Figure 6.1.

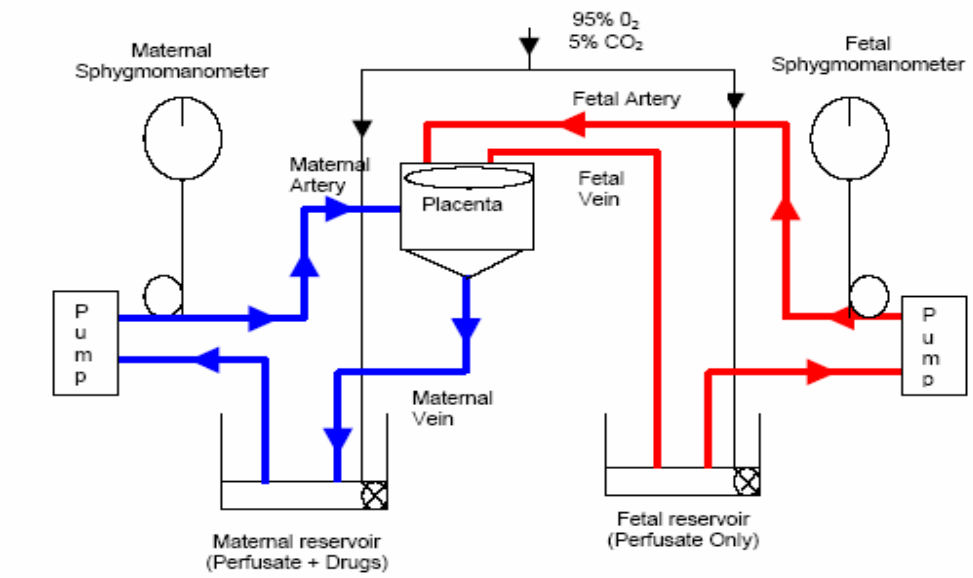


Figure 6.1: Schematic diagram of the *ex vivo* human placental perfusion model.

The use of human placental perfusions is an alternative or adjunct to animal testing and is preferable for two reasons. Firstly, because it is human based the outputs better reflect a human physiological response. Since the placental structure shows great interspecies variation (Leiser and Kaufmann, 1994), there are limitations in extrapolating placental

transfer data from animal studies to humans (Ala-Kokko *et al.,* 2000). Secondly, it avoids the ethical and financial difficulties associated with animal testing. One of the major disadvantages of an isolated organ perfusion is that absolute physiological conditions are not attainable. Also, the viability of the perfused tissue is limited.

The *ex vivo* human placental perfusion model was designed originally for the study of placental physiological features and has been applied to the transfer of electrolytes, amino acids, lipids and metabolites (eg Dancis *et al*.,1981, Schneider *et al.,* 1979, Dancis *et al*., 1974, 1976). More recently it is used to study drug transport (eg. Shiverick *et al.,* 2002, Heikkinen *et al.*,2001, Herman *et al.,* 2000) and aspects of the biochemistry of obstetrics (eg Huleihel *et al.*,2003) with the transfer of allergens being a more unusual application (Loibichler *et al.,* 2002). No-one to our knowledge is, or has, applied the model more widely to fetal exposure to environmental chemicals (Mitchell, personal communication).

In this chapter, potential fetal exposure to estrogenic compounds was investigated using *ex vivo* human placental perfusion. In the first instance, endogenous release of estrogenicity was determined in samples taken from both the maternal and fetal sides of the placenta over 4 hours. The background estrogenicity of these samples was measured using the yeast assay. Secondly, the endogenous hormone 17β-estradiol was added to the perfusate solution and samples collected and measured as for the blank perfusate. Thirdly, genistein was added to the perfusate, samples collected and analysed by HPLC. Details of the methodology are given in Chapter 8.6.

All perfusions were undertaken in collaboration with Mi-kyung, Iris Shin, Liggins Institute, University of Auckland, in fulfillment of her work for a Master's thesis (Shin, 2004). Analysis of the perfusates solutions was undertaken at ESR, Christchurch Science Centre, by the author.

6.2 Viability of placentas during perfusion

Biochemical and physical validation data for four perfusions are shown in Tables 6.3 and 6.4 with comparative data from Cannell *et al.,* (1988) (Shin, 2004).

Table 6.3: Physical and biochemical perfusion validation data with comparative data from Cannell *et al.*,1988.

	Experimental data from our studies			Comparative data[1]		
	mean	**SEM**	**n**	**mean**	**SEM**	**n**
Placental weight (g)	487	46	4	581	51	5
Weight of perfused mass (g)	18.6	0.16	4	21.7	6.0	5
Glucose consumption (mmol/kg/hr)	5.8	1.2	4	7.8	1.5	5
Lactate production (mmol/kg/hr)	7.1	1.6	4	8.5	1.4	5
hCG production (i.u./kg/hr)	2310	870	3	4860	3000	4
Fetal arterial pressure (mean mm Hg)	<40		4	<40		5
Average leakage (ml/hr)	<2		4	<2		5

Table 6.4: pH, pressure and flow rate parameters for perfusions.

	pH	P_{CO2} (kPa)	P_{O2} (kPa)	Pa (mmHg)	Flow rate (ml/min)
Maternal	7.48 ± 0.16	1.87 ± 0.54	28.88 ± 2.8	25.12 ± 7.62	10
Fetal	7.43 ± 0.21	1.06 ± 0.25	24.48 ± 1.58	35.2 ±4.01	4

The physical viability criteria for a successful perfusion was a pressure of less than 40 mmHg in the fetal circuit (to minimize shear stress), less than 2ml/hr leakage from the fetal to the maternal circuit, pH maintained between 7.2-7.4 and visual bleaching as the perfusion continued (Miller *et al.,* 1985, Cannell *et al*.,1988*).* Perfusions were rejected if these parameters were not matched. The average leakage of 2 ml/hr indicated that the respective circuits were intact and separate. For unsuccessful perfusions, the leakage was >10 ml/hr and these perfusions were discarded. Slightly higher pressures (up to 45 mmHg in the fetal artery) and leakages rates (up to 3 ml/hr) were tolerated in the 4th hour of perfusion.

Glucose consumption, lactate production and secretion of human chorionic ganadotrophin (hCG) into the maternal circulation showed that the placentas were intact and viable. Glucose consumption steadily declined in both the maternal and fetal compartments over the 4 hr duration of the perfusion. Lactate concentration in the maternal arterial

perfusates increased from 0.7 to 5.0 mmol/l over the 4 hours. The production of hCG over the 4 hours of perfusion was indicative of cellular metabolic activity. Stable PO_2 and PCO_2 values were maintained on both sides throughout the dual perfusions.

6.3 Endogenous estrogenicity

Because the placenta produces estradiol endogenously it was important to measure estrogenicity produced by the placenta and to determine the placental transfer of any estrogenicity produced, using the model system.

Two placentas were successfully perfused with perfusion medium and samples taken from both the maternal and fetal sides at time 0, 0.5, 1,2, 3 and 4 hours. The samples were assayed using the yeast assay and are shown in Figure 6.2 A,B.

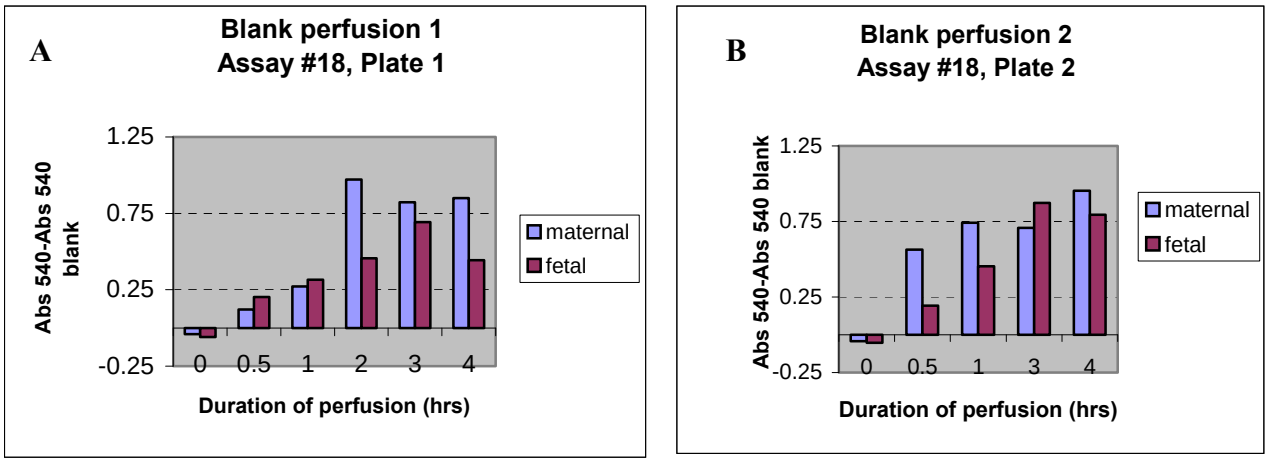


Figure 6.2 A,B: Estrogenicity of maternal and fetal samples following blank perfusions.

The assay showed an increasing amount of estrogenicity in both the maternal and fetal perfusate samples with time for the two placentas. This demonstrates that an endogenous estrogenic compound, or compounds, are being eluted from the placenta.

The blank perfusate solutions were subsequently re-analysed, following 6 months storage at -18˚C. The results obtained are shown in Figure 6.3 A,B.

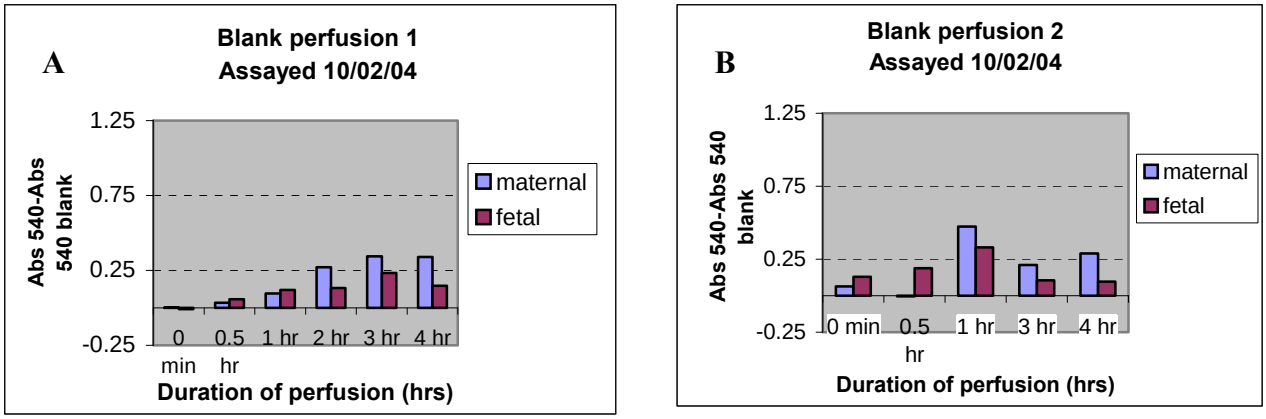


Figure 6.3 A,B: Estrogenicity of maternal and fetal samples following blank perfusions re-analysed after 6 months storage at -18˚C.

The apparent decrease in estrogenicity between the two assays is due to the difference in relative response to estradiol. This is seen both in the standard curves obtained (Figure 6.4) and the absorbance readings at 610 nm that are an indicator of cell growth.

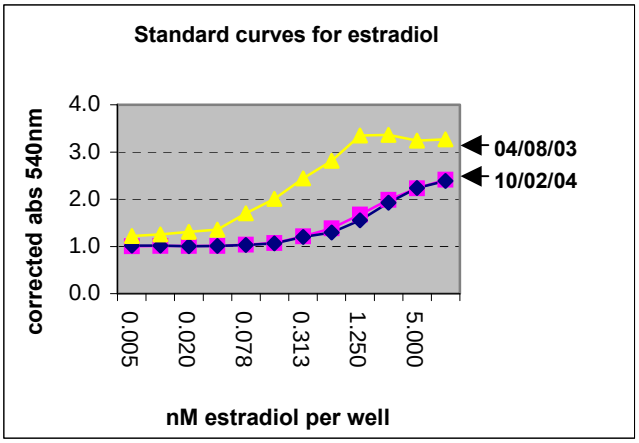
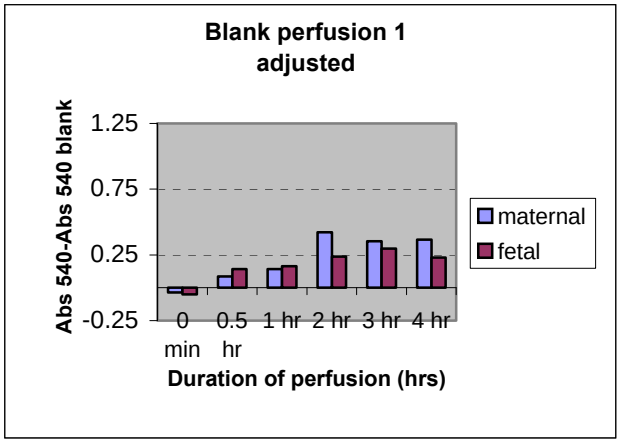


Figure 6.4: Standard curves for estradiol obtained from two different assays 6 months apart.

When the initial blank perfusion results are adjusted for the difference in response to estradiol between the two assays, much closer agreement between the two perfusions is observed (Figure 6.5 A,B).



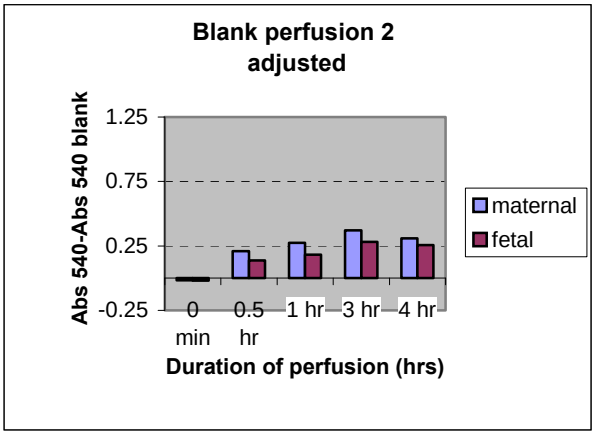


Figure 6.5: Initial blank perfusate assay results (04/08/03) adjusted for difference in response to estradiol (10/02/04).

The blank perfusates solutions developed estrogenicity within 0.5 hr that reached a plateau after 2 hours at a corrected absorbance (540nm) of about 0.25 in the perfusate solutions. The similarity of concentration in both the maternal and fetal sides suggests ready transfer, or equal production, on both sides of the placentae. The estrogenicity seen in the blank perfusion samples most probably does not reflect a physiologic condition since the substrates for placental estrogen synthesis (dehydroepiandrostendione and androstenedione) were not included in the perfusates solutions. However, the blank perfusions provide a baseline level of estrogenicity for the estradiol perfusions.

6.4 Placental transfer of 17β-estradiol

Three fresh placentae were perfused with 17β-estradiol at a concentration of 1µg/l. This concentration was selected as close to the circulating level in early pregnancy (Table 6.2) during the period of sexual differentiation and development of endocrine organs. This may well be a critical period for exposure to xenoestrogens. This concentration was also within the operating range of the yeast assay. Varying numbers of maternal and fetal samples were obtained, depending on the performance of the placenta, and analysed in duplicate for estrogenicity using the yeast assay in the same way as for the blank perfusions. The difference in estrogenicity between the maternal side and the fetal side is clearly seen in Figures 6.6 and 6.10.

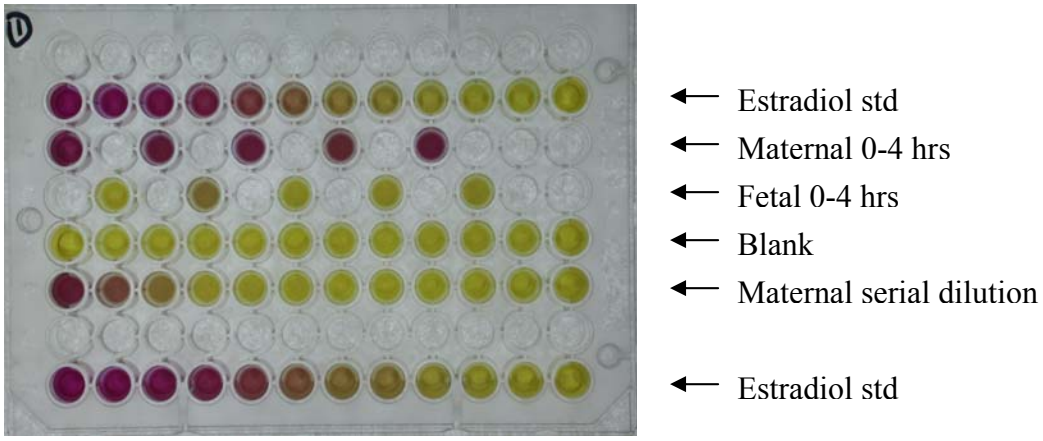


Figure 6.6: Yeast assay plate of perfusates from estradiol-perfused placenta

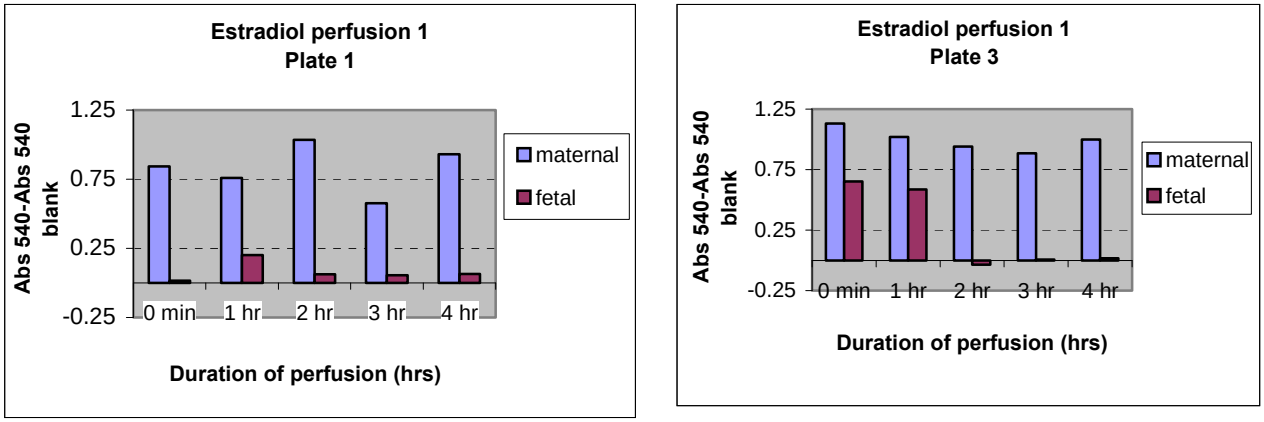


Figure 6.7 : Duplicate yeast assay results of estradiol perfused placenta 1

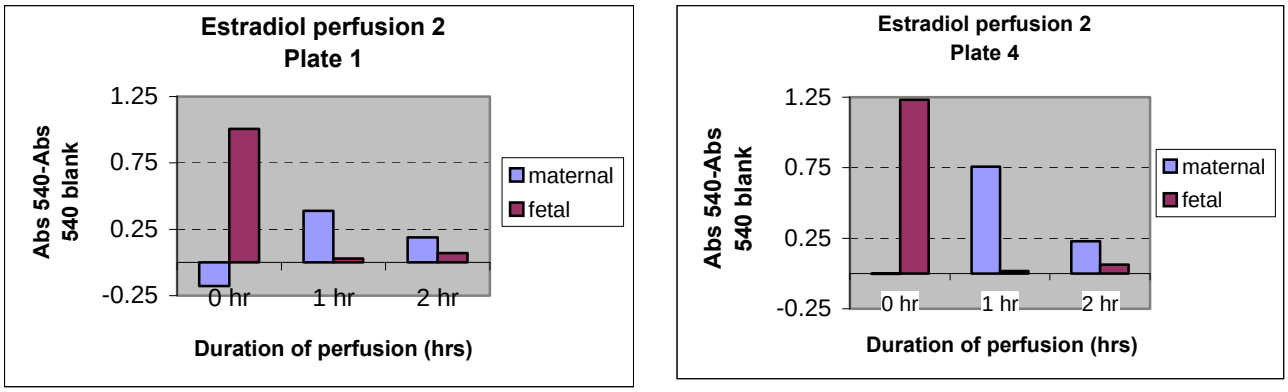
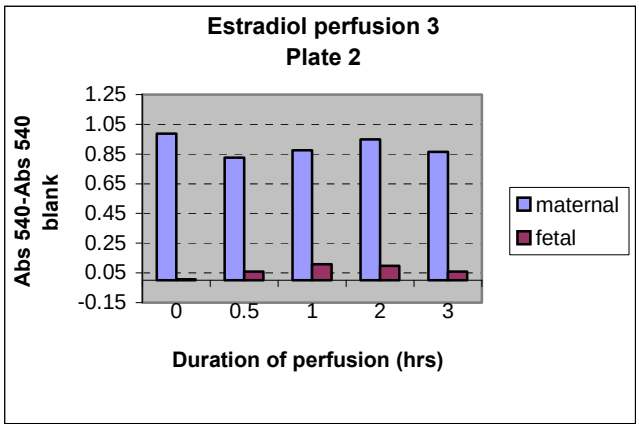


Figure 6.8: Duplicate yeast assay results of estradiol perfused placenta 2



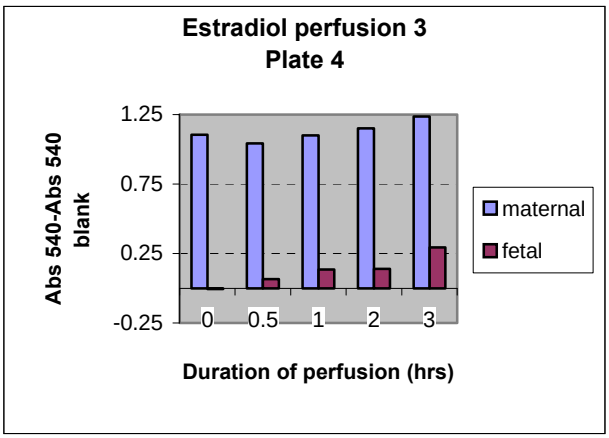


Figure 6.9: Duplicate yeast assay results of estradiol perfused placenta 3

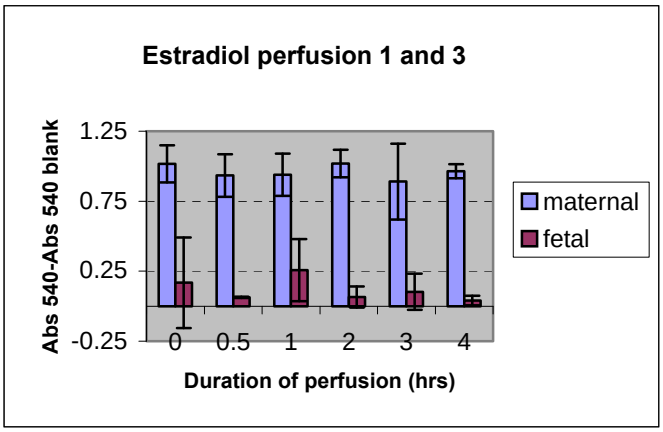


Figure 6.10 : Mean of estradiol perfusions 1 and 3.

Two perfusions of estradiol were successfully achieved and the results from these were combined and are shown in Figure 6.10. The third perfusion (Figure 6.8) leaked and therefore these results were excluded. From Figure 6.10 it is apparent that estrogenicity on the maternal side of the placentae was maintained for the 3-4 hours of the perfusion. There was no observable decrease in the level of estrogenicity on the maternal side nor a corresponding increase on the fetal side of the placentae. The implication of this finding

is that the placenta provides an effective barrier against *in utero* exposure to the endogenous hormone 17β-estradiol.

6.5 Placental transfer of genistein

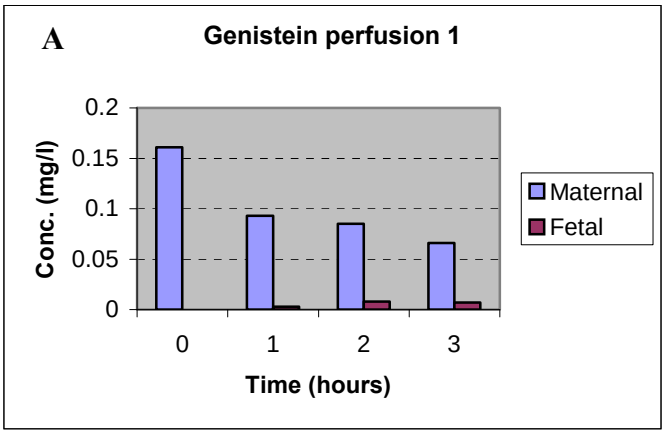
Three placentae were perfused with genistein at a concentration of 0.200 mg/l. This is about twice the mean serum level of 0.110 mg/l reported for Japanese women but well within the range of 0.001-0.680 mg/l reported in that study (Uehar *et al.*, 2000). Therefore it represents a realistic human exposure.

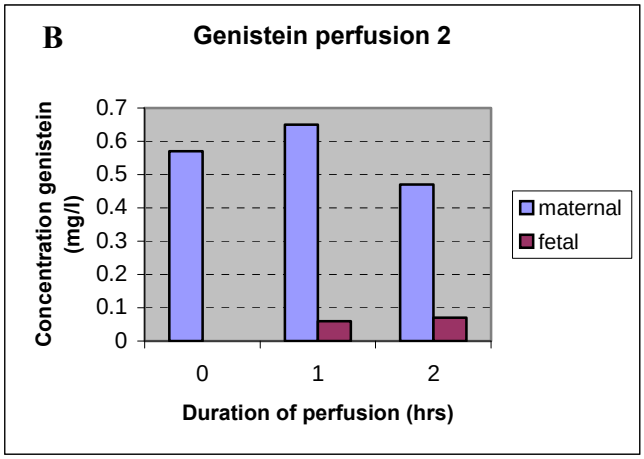
The concentration of genistein in the maternal and fetal perfusates samples was determined by HPLC. Details of the methodology are described in Chapter 8.6.5. The results are shown in Table 6.5 and Figure 6.11.

Table 6.5: Concentration of genistein in maternal and fetal samples of three perfused placentae (mg/l).

	Perfusion 1			Perfusion 2			Perfusion 3			mean
Hours	M	F	F/M	M	F	F/M	M	F	F/M	F/M±sd
0	0.161	0.001*		0.57	0.01	0.02	0.56	0.01	0.02	0.01 ±0.006
1	0.093	0.003	0.03	0.65	0.06	0.09	0.45	0.02	0.04	0.05±0.03
2	0.085	0.008	0.09	0.47	0.07	0.15	0.28	0.07	0.25	0.16±0.08
3	0.066	0.007	0.11				0.23	0.08	0.35	0.23±0.17

* = limit of detection, F= fetal perfusates, M = maternal perfusate





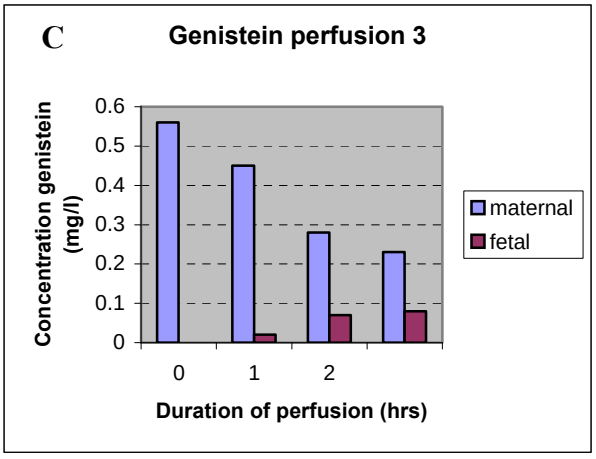


Figure 6.11: Concentration of genistein in maternal and fetal samples from three genistein perfusions, A,B,C.

The concentration results for the maternal samples of the perfusions undertaken on 23/04/04 and 06/05/04 are higher than is possible if spiked at 0.2 mg/l. Inspection of the HPLC chromatograms confirms the numbers shown in the graphs. The most likely explanation is that the latter perfusions were spiked at a higher concentration than 0.2 mg/l.

A small amount of genistein was detected in the fetal samples indicating that some placental transfer was occurring. Since the flow rate and volume of perfusates is greater on the maternal side (10 ml/min, 200ml) compared with the fetal side (3 ml/min, 100ml) it is expected that the concentrations on the two sides of the model are unequal. The percentage transplacental transfer from the maternal to the fetal side may be calculated:

$$\% \text{ TPT} = \frac{F_c \times F_v \times 100}{(F_c \times F_v) + (M_c \times M_v)}$$

Where: Fc = concentration in fetal perfusate sample at time x min, F_v = volume of fetal perfusate, : Mc = concentration in maternal perfusate sample at time x min, M_v = volume of maternal perfusate, (Henderson *et al*., 1992).

The % transplacental transfer of genistein for each of the 3 perfusions is shown in Table 6.6.

Table 6.6: Maternal to fetal transplacental transfer of genistein (%)

Hours	Perfusion 1	Perfusion 2	Perfusion 3	mean
0	0.3	0.9	0.9	0.7 ± 0.3
1	1.6	4.4	2.2	2.7 ± 1.5
2	4.5	6.9	11.1	7.5 ± 3.3
3	5.0		14.8	9.9 ± 6.9

6.6 Discussion

Application of the yeast assay with the placental model, demonstrates a barrier provided by the placenta for *in utero* exposure to estrogenicity from the endogenous hormone, 17β-estradiol. As far as the author is aware, this is the first application of the placental model to the study of transfer of estrogenic compounds from mother to the developing fetus. Although perfusions were undertaken at only one estradiol concentration, and serum estradiol concentrations span two orders of magnitude during pregnancy (0.1-30µg/l), the perfusion concentration was selected to approximate maternal serum levels when sexual differentiation and endocrine development of the fetus is occurring. This stage of pregnancy is likely to be a critical period of *in utero* exposure to hormonal compounds and the maternal serum concentration at this time is therefore an appropriate and interesting concentration to use for initial studies.

The yeast assay is designed to assess relative estrogenicity rather than for quantitation of absolute concentrations (because of the non linear, sigmoid nature of the standard curve). Therefore whilst the yeast assay will measure combined estrogenicity from different compounds, relative to a reference compound, it lacks precision to allow quantitation of placental transfer parameters. A more specific analysis is required.

The mean % transplacental transfer of genistein at 2 hours of perfusion (7.5 ± 3.3) is similar to the transfer of the antidepressant drugs, amitriptyline (8.2%) and nortriptyline (6.5%) (Heikkinen *et al.*, 2001) and the antibiotic drug acyclovir (10.2 %) (Henderson *et*

al., 1992). The transfer of genistein is less than for nicotine where about 19% of nicotine added to the maternal perfusates was transferred to the fetal perfusates and the fetal/maternal concentration ratio reached 1.0 (Rama Sastry *et al*., 1998). The mean transfer of genistein in the three perfusions was about one third of that measured by Henderson and colleagues (1992) for the fully diffusible marker, antipyrine.

Myllynen and Vähäkangas (2003) performed 6 perfusions with the drug diazepam at 2 and 0.2 mg/l, and the reference compound antipyrine that readily transfers across the placenta. At 2 hours the fetal-maternal ratio for diazepam was 0.48 and 0.55 (for 2.0 and 0.2 mg/l respectively) and 1.02 for antipyrine. Our studies show much less placental transfer for genistein, with a mean fetal-maternal ratio of 0.16 ± 0.08 at 2 hours (Table 6.5). This suggests that the placenta limits but does not completely inhibit transfer of genistein at dietary levels. Interestingly, there is some variance between this result and the findings of Adlercreutz *et al.,* 1999 who reported "ready transfer" of genistein from Japanese mothers to neonates on the basis of chord and amniotic fluid analysis (Adlercreutz *et al.,* 1999). They reported a median concentration of total isoflavonoids in the amniotic fluid of 7 subjects as 34nM compared with a median maternal chord plasma concentration of 147 nM. Thus the level in the amniotic fluid was 23% of the level in the chord plasma. The results reported by Adlercreutz and co-workers were for a full term pregnancy when the fetal metabolism is much more developed than at the period when exposure may be the most critical, namely 5-8 weeks. A similar lack of correlation between in vivo and placental perfusion data has been reported for diazepam (Myllynen and Vähäkangas, 2003). The placental model is a simplification of an *in vivo* system as it does not allow for fetal metabolism. This is an advantage for unravelling placental transfer versus fetal metabolic effects but a disadvantage for understanding the role of the placenta in regulating fetal exposure. It means that placental perfusion results need to be one of a number of methods used for investigating *in utero* exposure.

The three genistein perfusions show some variability in degree of barrier provided by individual placentas and hence the need for replicates. There is an apparent increase in concentration of genistein in the fetal samples with the corresponding decrease in concentration in the maternal samples for the perfusion 3. However, a placental barrier was maintained in each perfusion with the fetal to maternal concentration ratio reaching a

maximum of 0.35 and the maximum transfer being 15%. These results suggest an *in utero* exposure to genistein of between 5-15% of the mother's serum level.

There was a 40% decrease in genistein concentration in two perfusions 1 and 3 over the three hours of perfusion with a lower increase in concentration on the fetal side suggesting that genistein is being metabolized in the maternal circuit.

Due to the difficulty of obtaining placentas, it was not possible to undertake perfusions with bisphenol A. This would provide a most useful comparison of placental transfer of an endogenous estrogenic compound (17 β-estradiol) with a naturally occurring (genistein) and a synthetic estrogenic compound (bisphenol A).

Exposure to xenoestrogens at critical periods of development is a priority for research into the human health effects of EDCs. Since sexual differentiation and reproductive development occurs at 5-8 weeks gestation, and is permanently influenced by hormones, placental transfer of xenoestrogens is an important area of study. Although dual perfusions of human placenta are challenging to perform because of the difficulty in obtaining suitable placentae and the delicate nature of the tissue that is prone to leakages, this technique is an interesting way of gaining a better understanding of relative placental transfers of xenoestrogens from mother to fetus.

6.7 Summary

Seven dual perfusions were successfully undertaken.

A small amount of estrogenicity is produced by the human placenta in the model system and this transfers freely between the maternal and fetal sides.

Estradiol added to the maternal perfusate is maintained over 4 hours of perfusion, with little transfer to the fetal side.

A small amount of genistein, in the order of 5-15% is transferred from the maternal to fetal sides of the placenta using the dual perfusion model. This is similar to the transfer of

a number of drugs (amitriptyline, nortriptyline and acyclovir) but less than for the reference compound antipyrene, the drug diazepam, and nicotine. The results suggest that the placenta limits transfer of genistein at dietary levels in the human system. A possible discrepancy with *in vivo* data means that this placental transfer model must be considered with caution.

The placental model provides an interesting tool for assessing the relative transfer of xenoestrogens across the placenta to add to our knowledge of fetal/*in utero* exposure to these compounds at an important life stage. Further studies of other xenoestrogens will provide a basis for comparison.

Chapter 7

A preliminary genomic approach to investigating the health effects of xenoestrogens

7.1 Introduction

A range of adverse human health effects that have been associated with endocrine disrupting chemicals, of which xenoestrogens are a subset, are described in Chapter 1.3.2 including:

- Enhancement of carcinogenesis (breast, uterus, ovary, prostate, testis)
- Elevated carcinogenic potential in the 2nd generation (vagina, breast, uterus, prostate, testis)
- Reproductive dysfunction (spermatogenesis, sexual cycle, endometriosis, fertility)
- Developmental disorders of sexual organs (reproductive organ malformation, hypoplasia)
- Precocious puberty
- Immune toxicity (lowered resistance to infection)
- Neurotoxicity in the 2nd generation (growth retardation, impairment of intelligence, emotional instability (WHO, 2002a, Shirai and Asamoto, 2003, emcom, 2004).

The assessment of dietary exposure to xenoestrogens (Chapter 2) implicates a possible pharmacological effect of dietary xenoestrogens for adult males. Of the adverse effects above, those that might plausibly result from male dietary exposure are enhancement of

prostate and testicular cancer, reproductive dysfunction, and immune toxicity. Causal associations, measured as a difference in odds ratios between case and control subjects, are relatively attainable. An example is the significantly increased risk of cryptorchidism found in the sons of women working in gardening (adjusted odds ratio=1.67, 95% confidence interval 1.14-2.47 (Weidner *et al*, 1998)). This study analysed the incidence of 6177 cases of cryptorchidism in Denmark during the period 1983-1992 with 23,273 control subjects against risk factors including parental occupation. An adjusted odds ratio greater than 1.0 suggests a positive association between two variables. The greater the number, the stronger the association. However, proving that exposure to a chemical in food causes a chronic health effect, such as prostate or testicular cancer, is notoriously difficult because of the delay between exposure and detection of the disease state.

Emerging "omics" technologies offer an exciting opportunity to substantiate cause and effect of environmental hazards that are mitigated by altered gene expressions.

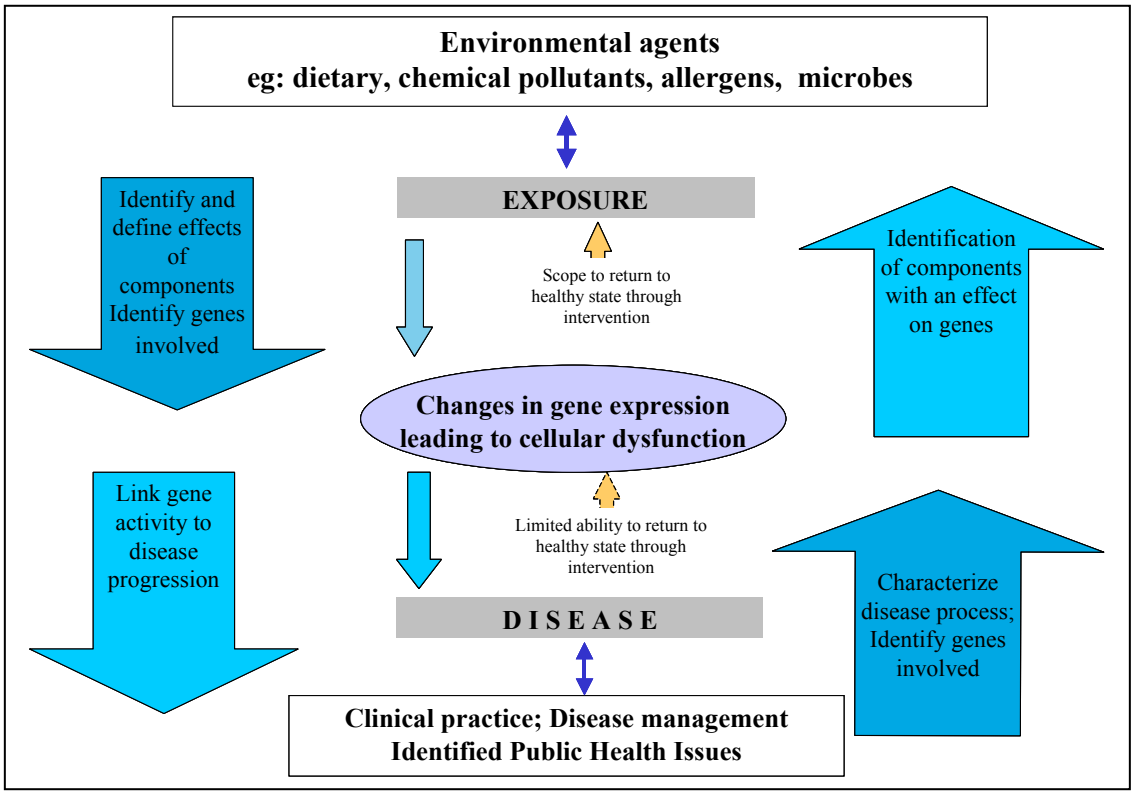


Figure 7.1: Schematic of the connection between exposure, gene expression and health outcome

Using mini or microarrays it is possible to simultaneously screen the expression of thousands of genes. This methodology has been applied to the identification of estrogen

responsive genes in breast cancer cell lines (Coser *et al*., 2003, Inoue *et al*., 2002, Hayashi S-I, 2004, Soulez and Parker 2001, Terasaka *et al*., 2004) but not yet of either prostate or testicular cell lines or tissues. If indeed, exposure to xenoestrogens leads to breast, prostate and testicular cancers via ligand binding to the estrogen receptor, and a transcriptional pathway, it follows that the resulting gene, enzyme or protein expression profiles would be different for cells of these types that are exposed compared with non-exposed cells. The relative response, either 'up' or 'down' regulation, of the genes, enzymes and proteins involved is measureable using '-omic' technologies. Moreover, if different xenoestrogens act by the same mechanism and are additive, (Silva *et al.,* 2002) gene expression profiles for different xenoestrogens will be the same. This has not yet been demonstrated. An understanding of how gene expression is altered by xenoestrogens at a cellular level, mapped against emerging information on the progression of hormone cancers will provide knowledge about the etiology of these cancers and guidance for therapeutic and intervention strategies.

7.1.1 Evidence for a role of xenoestrogens in prostate cancer

There are racial differences in susceptibility to prostate cancer, with the incidence being rare in Asians, higher in Caucasians and higher still in African-American males (Crisp *et al*., 1998). A racial difference in prostate cancer incidence is suggestive in New Zealand with the incidence rate, based on cancer registrations, for Maori men approximately half that of the total New Zealand male population (65 and 117 per 100,000, age standardized, respectively) (NZHIS, 2004). Racial differences are likely to be a combination of genetic (Carter *et al*., 1993), age related and environmental factors. The rate of prostate cancer incidence increases with age as the ratio of estrogen to testosterone increases (Ho, 2004), consistent with an association between estrogenic compounds and prostate cancer. A comparison of prostate cancer incidence rates between migrants and residents of the country of origin, supports a role of environmental or lifestyle factors in prostate cancer incidence (Doll and Peto, 1981).

Two recent epidemiological studies report a higher incidence of prostate cancer in agricultural workers. The incidence of prostate cancer among commercial agricultural workers was 40% higher than predicted on the basis of state age-adjusted incidence rates

for a cohort of 55,332 male Americans in the Agricultural Health Cohort Study (Alavanja *et al*., 2003). Chlorinated pesticides (aldrin, chlordane, DDT, 2,4,5-T were significantly associated with the increased incidence of prostate cancer. Additional follow-up studies for this cohort are anticipated to further strengthen the association between farming and prostate cancer risk. In a case-control study undertaken in Italy, a 40% increased risk of prostate cancer was associated with agricultural workers (Settimi *et al.,* 2003). Major components of pesticides associated with an increased risk of prostate cancer included organochlorines, specifically DDT and combined treatments of dicofol and tetradifon. The mechanism of action for prostate cancer remains to be elucidated but an endocrine pathway is one possibility.

Various associations between hormone levels and prostate cancer incidences give a confusing picture for a role for estrogenic compounds in the aetiology of prostate cancer. For example, African-Americans, who have the highest incidence of prostate cancer in the USA, also have significantly higher levels of serum estrogens, even at a young age, when compared with their Caucasian-American peers (de Jong, 1991, Ho, 2004 and references therein). Consistent with high serum estrogen levels being a risk factor for prostate cancer, lower circulating estrogen and testosterone levels have been noted in Japanese men who have a low rate of prostate cancer compared with higher risk Caucasian-Dutch men (de Jong *et al*., 1991). Conversely, levels of soy-derived estrogenic compounds in the prostatic fluid of Asian men, with higher soy consumption (but lower prostate cancer incidence), are up to 17 times higher than Western men (Morton *et al.,* 1997), evidence that diet may influence circulating 'estrogenicity' levels. Further, the ability to produce equol (a metabolite of the isoflavone daidzein) is closely associated with a lower incidence of prostate cancer (Akaza *et al.,* 2004). The protective and/or risk nature of circulating estrogens remains to be clarified.

There are at least two plausible theories to explain the mechanism of action of estrogenic compounds and prostate cancer (Ho 2004). The first theory is that cancer results from DNA damage, either directly via estrogen metabolite-DNA adducts or indirectly via the formation of reactive oxygen species. An alternative theory is that cancer results from enhanced cell proliferation via ER binding. There are two ER subtypes in humans, ERα and ER-β, and estrogenic ligands bind with different affinities to the two receptors (Kuiper *et al*., 1997). Both subtypes have been identified in normal human prostate cells

although the localization of the two subtypes differs (Latil *et al*., 2001, Leav *et al*., 2001). ER-β but not ER-α is strongly expressed in normal prostate cells (Lau *et al*., 2000). ER-β is thought to exert anti-proliferative effects in the normal prostate, thus being cancer protective (Zhu *et al*, 2004, Cheng *et al*., 2004). Expression of ERβ is lost as prostate cancer progresses (Latil *et al*., 2001, Lau *et al*., 2000), possibly by a DNA methylation pathway (Lau *et al*., Zhu *et al*., 2004).

Although ER β is more highly expressed in normal human prostate cells than ER-α , studies with ER-α and ER-β knock-out mice suggest that ER-α may be the dominant receptor mediating prostate development (Prins *et al*., 2001). At a genomic level, Cancel-Tassin and co-workers (2003) looked at polymorphisms of the human ER- α gene in healthy and prostate cancer cases. Their findings implicated variants of a GGGA polymorphism of the ER-α gene with an increased risk of developing prostate cancer.

Interestingly, estrogen therapy, is one treatment regime for prostate cancer. Charles Huggins pioneered the use of the synthetic xenoestrogen, diethylstilbestrol, for the treatment of advanced prostate cancer in the early 1940s. This treatment was subsequently discontinued because of adverse effects including feminization and heart failure (Huggins, 1941). With increasing health costs and improved cardiovascular care there is renewed interest in estrogen therapy for prostate cancer treatment (Oh, 2002, Messina, 2003). Estrogen analogs are also being synthesized as potential drugs for prostate therapy (Mobley JA *et al.,* 2004). Estrogenic drugs could plausibly treat prostate cancer by up-regulating expression, and hence antiproliferative effects, of ER-β that diminishes in cancer cells. However, experiments undertaken by Mobley and co-workers (2004) suggest that the estrogen analogue that they tested induced apoptosis through a non ER mechanism, even though it bound preferentially to ER-β. The protective association of equol (Akaza *et al.,* 2004) may be by the same mechanism as the estrogen analogue synthesized by Mobley and co-workers or by maintaining expression of ER-β.

7.1.2 Evidence for a role of xenoestrogens in testicular cancer

Cryptorchidism (undescended testes) is a known risk factor for testicular cancer. The significantly increased risk of cryptorchidism found in the sons of women working in

gardening (Weidner *et al*, 1998) implicates pesticide, and therefore possibly xenoestrogen, and a possible prenatal etiology with this condition (Moss *et al*., 1986, Henderson *et al*, 1988). Although individual studies of a link between maternal exposure to the synthetic estrogen, diethylstilbestrol, and an increase in the incidence of testicular cancer are variable (Henderson *et al*., 1988), a meta analysis of studies reported an overall increase of approximately twofold (Toppari *et al*., 1996).

The type of testicular cancer found in humans is very rare in laboratory animals and the lack of suitable animal model systems has limited the scope to prove a link between exposure to estrogenic chemicals and testicular cancer. There is limited data from animal studies that in utero exposure to a high level of estrogen may increase the risk of developing testicular cancer and that exposure of rabbits *in utero* and/or in infancy to octylphenol, *p,p'*-DDT/DDE or zeranol leads to pretesticular cancer cells (ICPS, 2002).

There have been a few reports of ER gene expression in human testicular cells that begin to provide a mechanistic link between exposure to estrogenic compounds and testicular cancer. ER-β and co-regulator mRNA levels have been compared in matched samples of human testicular tumors and adjacent normal tissues (Hirvonen-Santti *et al*., 2003). These results showed that expression of ER-β and one of the cofactors was down regulated in the cancerous compared with the normal tissue, suggesting that these genes may play a role in testicular tumorigenesis.

Gaskell *et al*., (2003) detected expression of ER-β but not ER-α in human fetal testis cells at 12-19 weeks gestation and suggested that estrogenic compounds may influence the genesis and progression of testicular cancers through their interaction with ER- β following in utero exposure to estrogenic compounds.

Pais *et al*., (2003) compared the localization of ER-α and ER-β receptors in normal and testicular germ cell cancers. ER-β, but not ER-α, was expressed in normal and testicular germ cell cancer cells suggesting that estrogenic effects on the testis are mediated only via the ER-β subtype. ER-β expression was down regulated in two forms of testicular cancer but remained high in endodermal sinus tumors and teratomas. The differences in ER-β expression levels in different testicular germ cell tumors may reflect divergent pathways of these neoplasms from a common precursor (Pais *et al*., 2003).

7.1.3 Additivity of estrogenicity

There is evidence for additive estrogenicity of low concentrations of various xenoestrogens from the work published by Payne *et al.,* (2000), Silva *et al.*, (2002), Rajapakse et al., (2002), Rajapakse *et al*., (2004) and Gaido *et al.,* (2003). Silva and colleagues (2002) combined eight xenoestrogens including hydroxylated PCBs, benzophenones, parabenes, bisphenol A and genistein at concentrations below individual No Observable Effect Levels in proportion to their individual potencies. This finding suggests a similar mechanistic pathway. Whether different xenoestrogens, in particular endogenous versus natural versus synthetic, alter gene and transcript expression in the same or different ways in human prostate and testicular cells remains to be elucidated.

7.2 Cell culture

As the first step of a genomic approach to testing the hypothesis that endogenous, naturally occurring and synthetic estrogenic compounds alter gene transcript expressions in the same way in prostate and testicular human cells, cell culture capability was developed under the guidance of Professor John Masters, at the Prostate Cancer Research Centre, University College London, UK. Time and budget constraints limited the amount of experimental work that could be achieved.

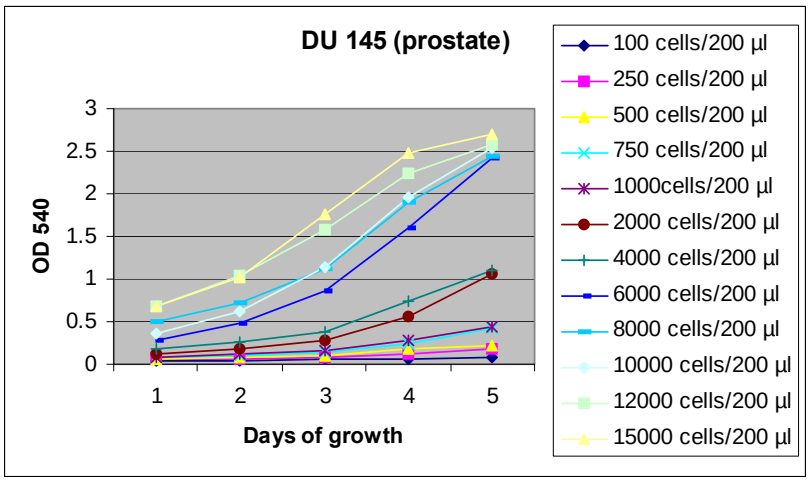
7.2.1 Growth curves

Prostate and testicular cell lines were targeted for study because these are male cells and males were signaled to be a higher risk priority than females from the risk assessment described in Chapter 2. Four human cancer cell lines, including DU 145 (prostate), SuSa (testicular), 833K (testicular), and HT29 (colon) were grown over 5 days (Chapter. 8.7.3). Five plates were prepared for each cell line and cell growth was measured in a different plate on days 1-5 using the colourimetric assay developed by Skehan *et al*., (1990). In this assay, cell growth is measured as cell protein, visualized by binding to a dye, sulforhodamine, and measuring absorbance at 540nm on a 96 well plate reader. Mean absorbance values were calculated for each column on the plate. The growth curves, Figure 7.1, show that each cell line grew exponentially over days 2-4 following a lag phase, especially evident for the solutions with low cell counts. A stationery growth

phase was reached by day 4 for the higher cell counts for all cell lines. The 833K cells were particularly prone to contamination leading to cessation of growth. The variability in cell growth was greatest for SuSa cells (Table 7.1).

Table 7.1: Variability of cell growth for DU 145, SuSa, 833K and HT 29 cell lines (%CV)

Cell Line	DU 145	SuSa	833K	HT29
Day 1	3.6-15.8	2.5-69.4	2.9-9.7	5.5-35.1
Day 2	2.4-19.7	3.6-59.7	1.3-15.5	2.0-27.9
Day 3	2.5-18.2	3.1-39.3	2.3-9.4	5.7-22.7
Day 4	2.7-12.5	2.7-59.8	4.1-16.1	0.7-12.2
Day 5	0.9-10.5	0.6-61.4	NR	0.4-17.5



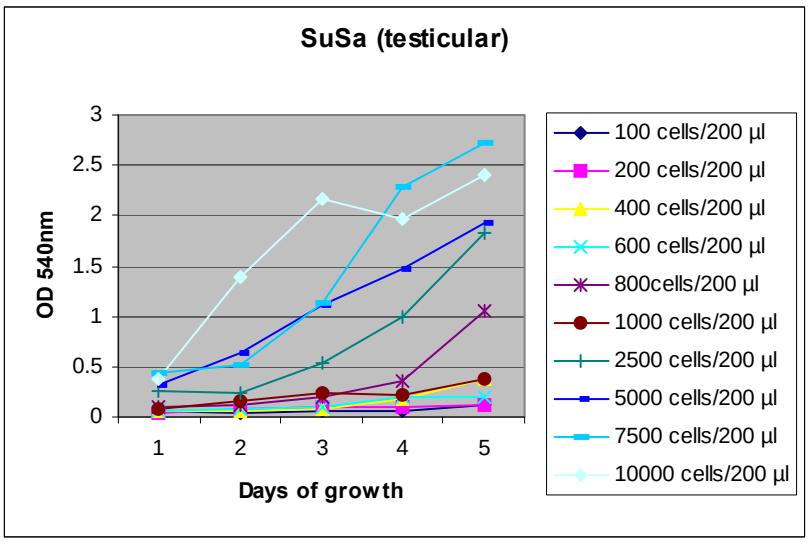
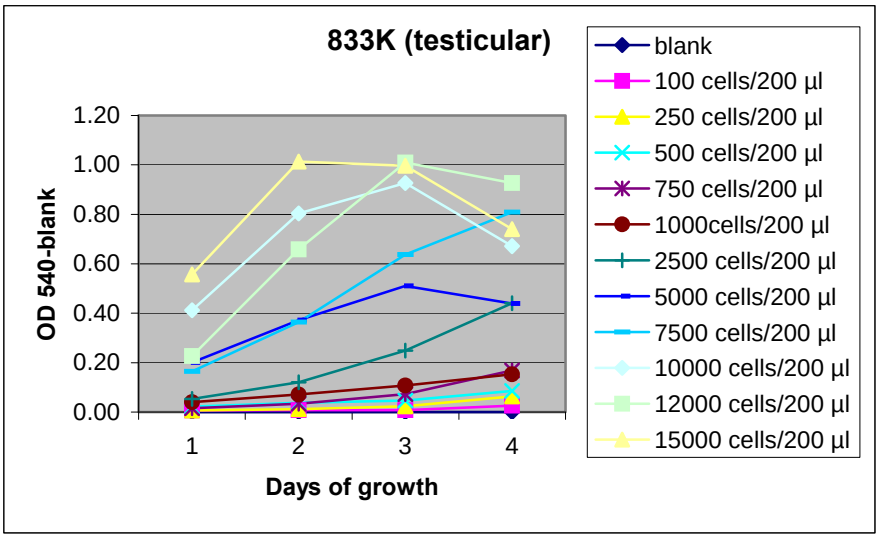


Figure 7.1a: Growth curves for a prostate (DU145) and testicular (SuSa) cell lines with varying initial levels of inoculation.



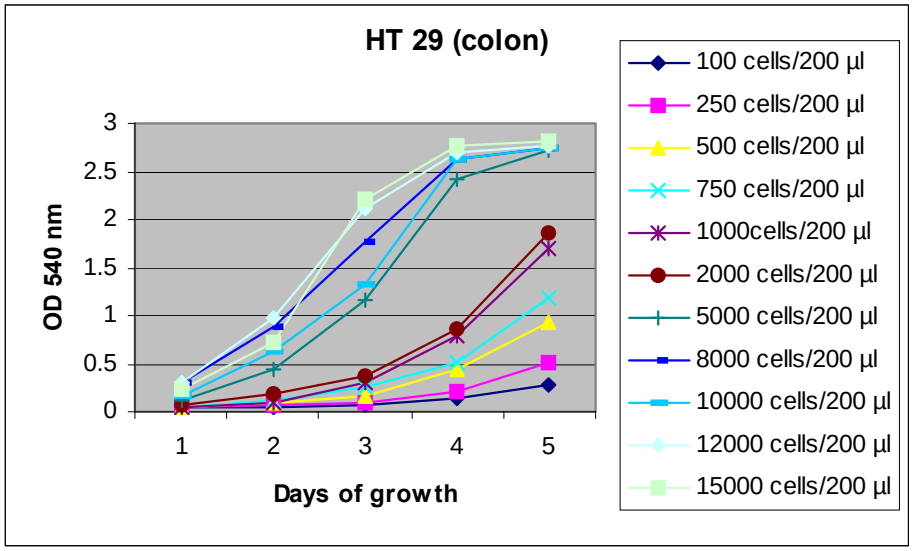


Figure 7.1b: Growth curves for a testicular (833K) and colon (HT 29) cell lines with varying initial levels of inoculation.

7.2.2 Impact of estrogenic compounds on cell growth

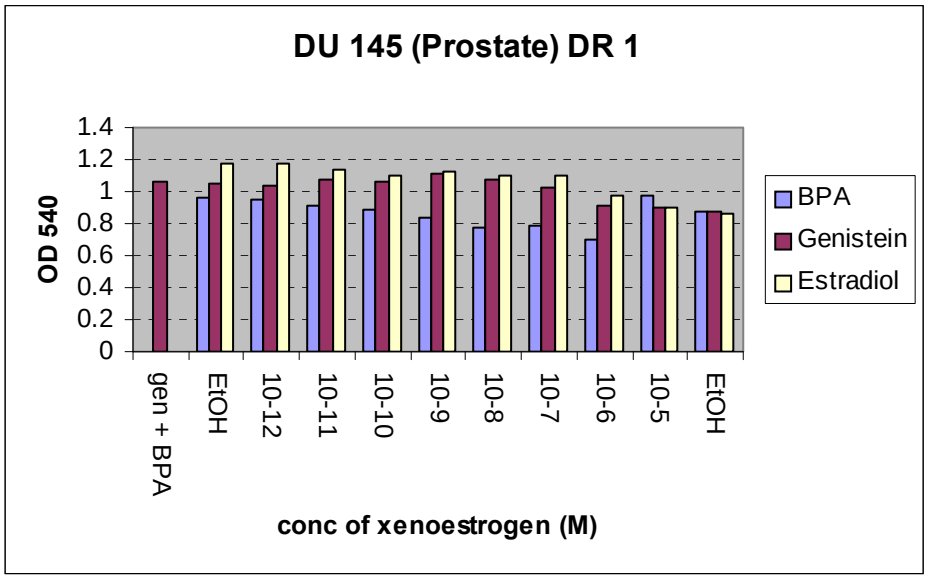
Information from the growth curves was used as a basis for designing dose response experiments. To gain an understanding of how selected estrogenic compounds might affect the growth of the cell lines of interest, duplicate dose-response experiments were carried out. Details of the methodology are described in Chapter 8.7.4, but in principle,

aliquots of cell culture were exposed to varying amounts of test compound and cell growth was measured after 3 days incubation. The human breast cancer cell line MCF-7 was included in these experiments as a positive control. Cell growth was determined by the SRB assay on day 3 and the results are shown in Figures 7.2 a-e.

The reproducibility of duplicate dose response experiments was not good but not unexpected. This was partly attributable to greater variability of replicates for Du 145, 833K and HT29 compared with the growth experiments (Table 7.2). An apparent decrease in cell numbers with increasing concentration of estrogenic compound across the plate is not real. There was a systematic error with the experimental setup because the control columns containing ethanol only, on either side of the plates were not consistent. Decreasing cell numbers were in the same direction as the addition of culture across the plate and therefore it seems likely that the 200μL aliquots of culture medium contained decreasing numbers of cells as aliquots were added across the plate.

A control column containing only culture and medium was included in the second dose response experiment. Comparison of cell growth for this column with the adjacent column containing ethanol, but no estrogenic compounds, shows that the ethanol itself is not toxic to the cells and the technique of adding a test reagent in ethanol and allowing this to evaporate, is practical and avoids the problem of solvent toxicity.

With one exception (HT29, DR 1), cell growth following exposure to an estrogenic compound, was within the range observed for the control wells across concentration range 10^{-5} to 10^{-12} M. There is no consistent evidence of toxicity across this concentration range. Human serum concentrations of 17β- estradiol, genistein and BPA are in the order of 10^{-10}M, $4x10^{-7}$M and 10^{-8} M respectively (Greenspan and Gardner, 2001, Uehar *et al.,* 2000, Takeuchi *et al,* 2002). These concentrations are mid range of the dose response experiments and therefore appropriate doses for future cell exposure experiments.



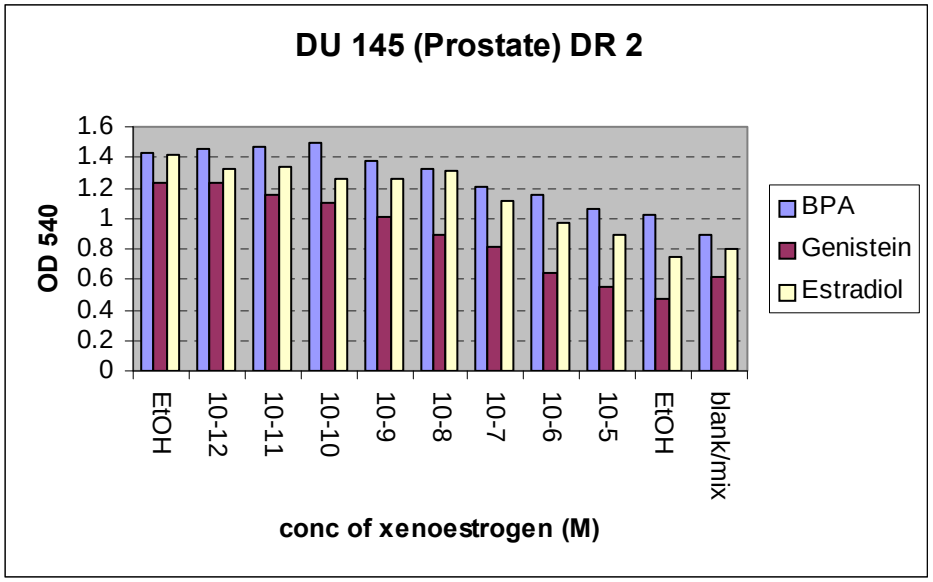
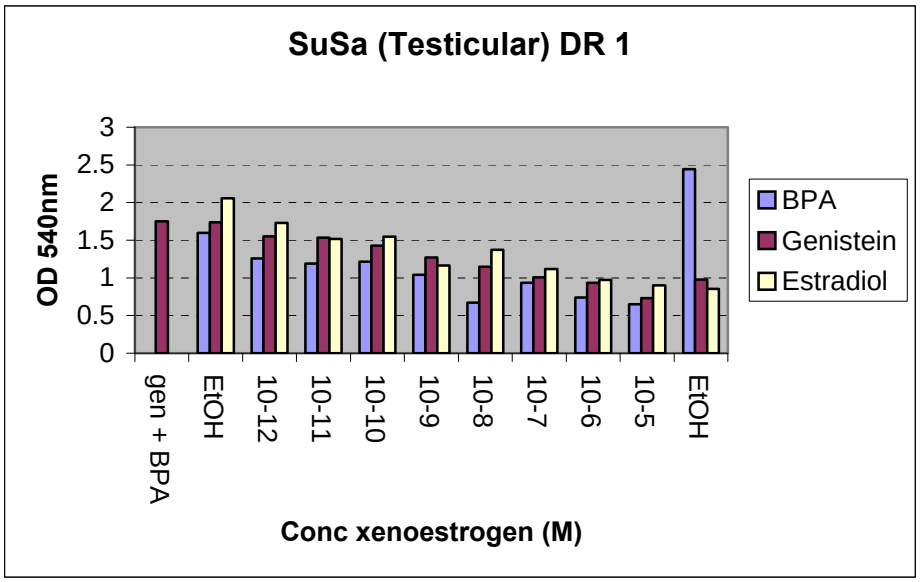


Figure 7a: Dose response curve of prostate cells (DU145 to BPA, genistein and 17β-estradiol



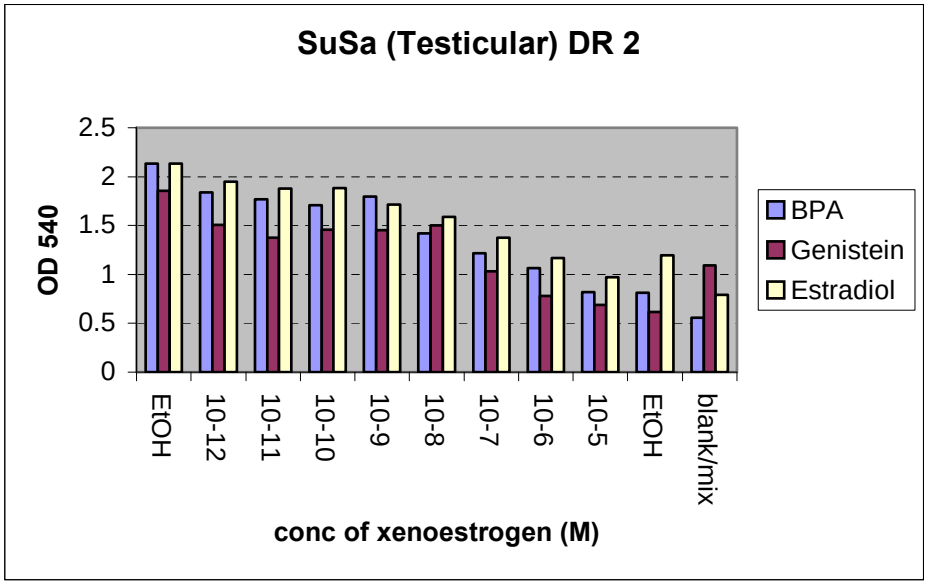
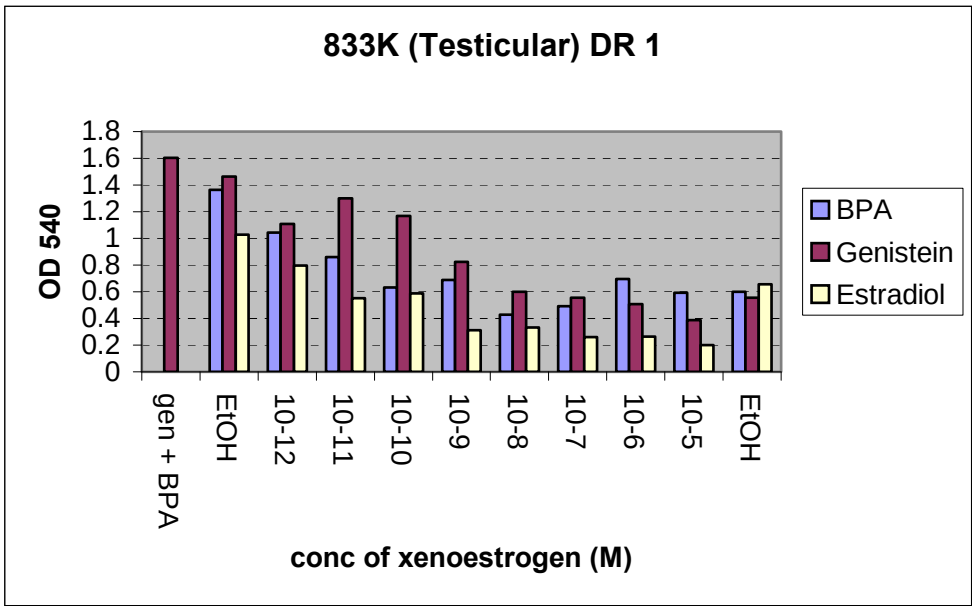


Figure 7b: Dose response curve of testicular cells (SuSa) to BPA, genistein and 17β-estradiol



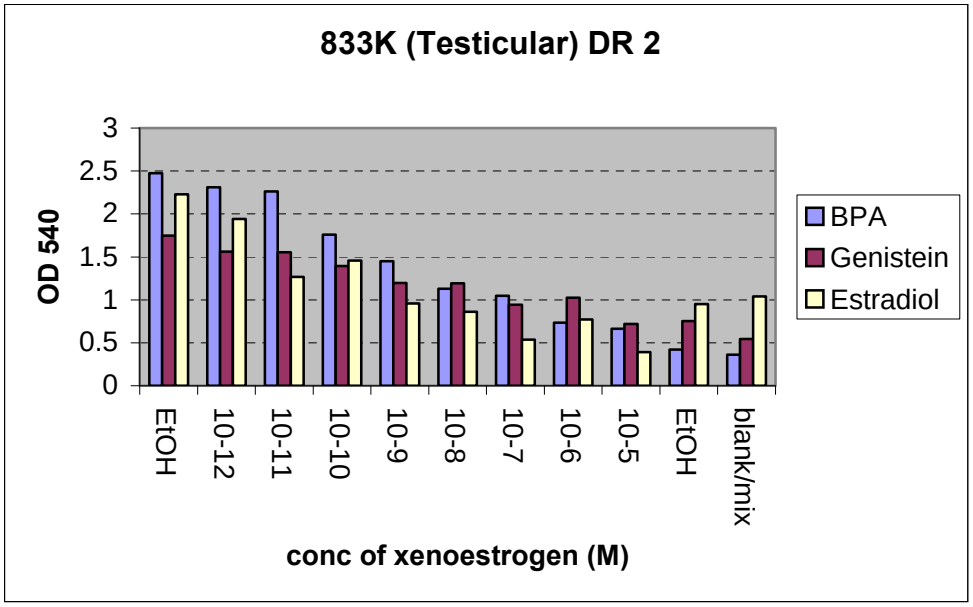
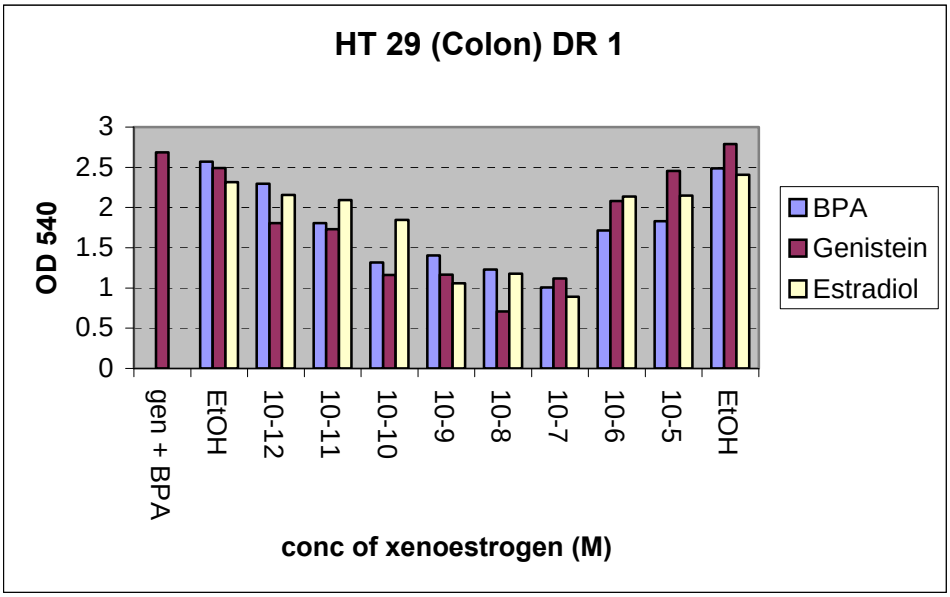


Figure 7c :Dose response curve of testicular cells (833K) to BPA, genistein and 17β-estradiol



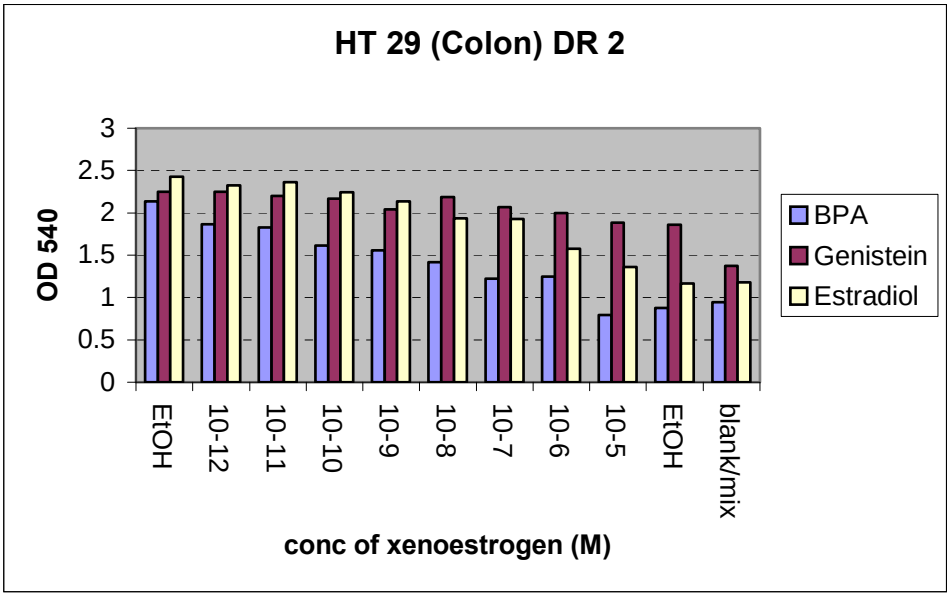
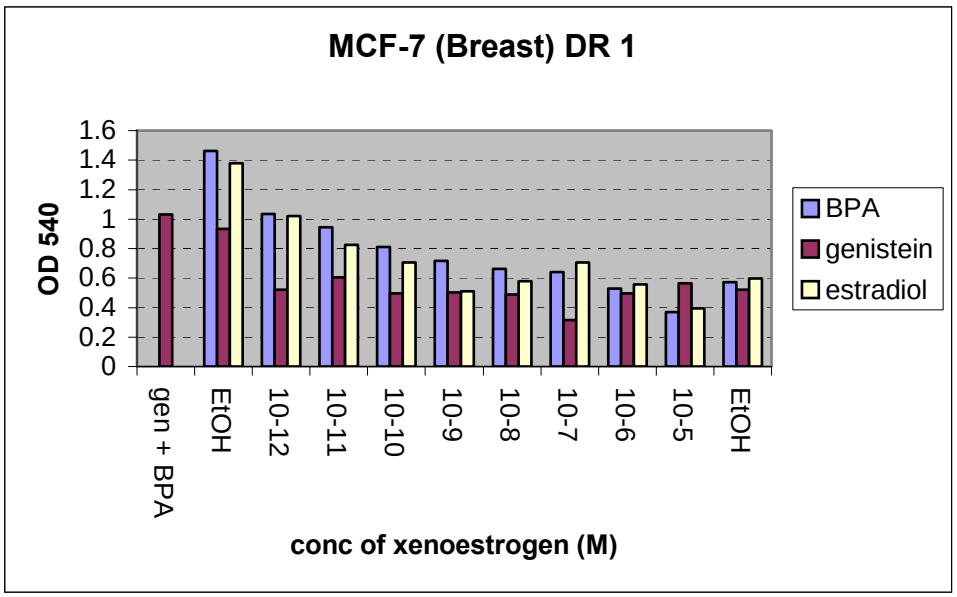


Figure 7d:Dose response curve of colon cells (HT 29) to BPA, genistein and 17β-estradiol



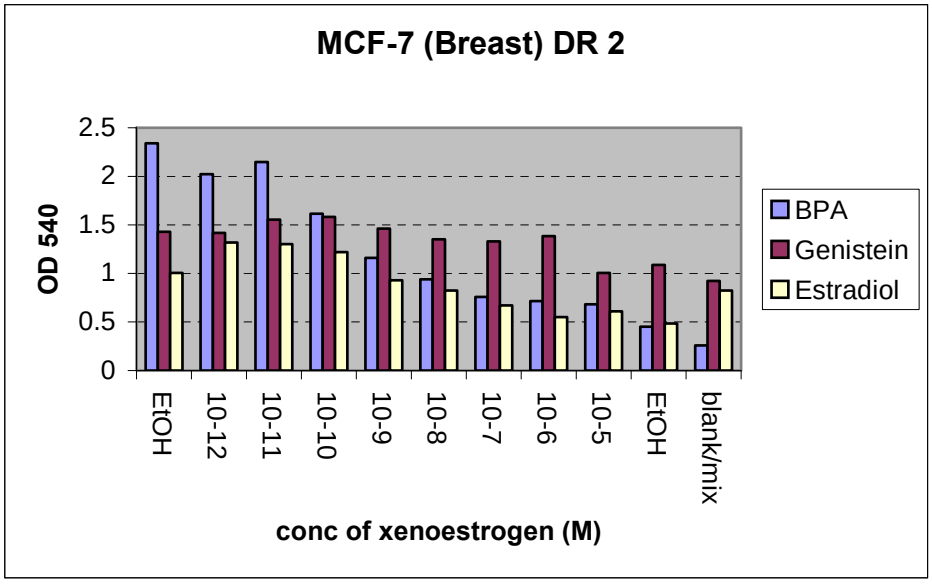


Figure 7 e: Dose response curve of breast cells (MCF-7) to BPA, genistein and 17β-estradiol

Table 7.2: Variability of cell growth of DU 145, SuSa, 833K, HT 29 and MCF-7 cell lines following exposure to 17β- estradiol (E2), the naturally occurring xenoestrogen genistein and the synthetic xenoestrogen BPA. (%CV).

	Experiment	DU 145	SuSa	833K	HT29	MCF-7
E2	1	3.2-14.7	4.6-16.5	2.8-40.6	1.8-47.5	2.2-23.7
	2	3.9-25.1	3.7-40.2	3.2-2.6.3	3.9-25.2	3.6-46.7
Genistein	1	4.1-12.9	4.6-15.7	3.7-32.6	1.6-53.7	2.2-64.6
	2	3.0-26.8	4.5-37.3	3.9-24.3	4.7-18.8	4.0-38.1
BPA	1	2.6-19.4	1.0-26.4	1.0-30.4	4.3-45.2	2.2-48.2
	2	4.7-14.8	1.1-27.8	1.9-49.2	1.6-21.5	4.2-61.9

7.3 Genomic analysis

Ideally target cell lines that had been exposed to estrogenic compounds would be screened for effect on the expression of a wide range of genes using DNA microarray techniques. A number of investigators have taken this approach to develop a library of estrogen responsive genes using breast and sertoli cell lines (Coser *et al*., 2003, Inoue *et al*., 2002, Soulez and Parker 2001, Tabuchi *et al*., 2002, Terasaka *et al*., 2004). However it was considered prudent to first confirm that the cells had absorbed the test compounds by measuring expression of ER-α and ER-β gene transcripts using real-time quantitative reverse transcription-PCR (Q, RT-PCR) (Latil *et al*, 2001, Lau, 2000, Pais *et al*., 2003).

7.3.1 Exposure of cells to estrogenic compounds

Five human cancer cell lines including prostate (DU 145), testicular (SuSa, 833K), colon (HT29) and breast (MCF-7) were exposed, in larger T-80 vessels, to either 17β-estradiol, genistein, bisphenol A or a combination of genistein and bisphenol A at concentrations approximating serum concentrations (1.8×10^{-10} M, 3.1×10^{-6} M, 1.2×10^{-7}M). Details are given in Chapter 8.7.6. Control cells that were not exposed were also maintained for each cell line.

Of the 25 cultures, 6 became contaminated with fungi. The 833K cells grown in the presence of BPA, both alone and in combination with genistein, did not grow, suggesting

that the this level of exposure was in fact toxic to the cells. Alternative control cells of HT 29 and SuSa were extracted and dispatched to New Zealand. Time and budget constraints precluded a repeat of this exposure but a useful set of exposed cells was achieved.

7.4 Discussion

The epidemiological data, the change in expression of ER-β with the progression of prostate cancer, the results of experiments with knock-out mice and the use of estrogen therapy for prostate cancer treatment support a role of ER mediated responses in human prostate cancer. Therefore naturally occurring and synthetic xenoestrogens that bind to, and influence the expression of ER-α and ER-β, are highly likely to be implicated, either as beneficial or risk factors in prostate cancer.

Both animal and human studies suggest that *in utero* exposure to estrogenic compounds may influence the genesis and progression of testicular carcinogenesis through interaction with ER-β.

There is no evidence to confirm similar, or different, effects of endogenous and naturally occurring or synthetic xenoestrogens, on the expression of ER subtypes in human prostate or testicular cells.

7.5 Summary

Cell culture capability was developed by maintaining and exposing five different human cancer cell lines to three test estrogenic compounds. These cell lines reached exponential growth in 3 days following inoculation at 35,000 cells/ml. There was no consistent evidence of toxicity of 17β- estradiol, genistein and BPA over the concentration range 10^{-5} to 10^{-12} M. The levels found in human serum are about mid range and therefore this level of exposure is unlikely to adversely affect cell growth. Analysis for changes in expression of ER-α and ER-β receptors following exposure is yet to be completed.

Chapter 8

Experimental

A quote:

"The work of science is to substitute facts for appearances, and demonstration for appearances"

-John Ruskin, The Stones of Venice.

8.2 Experimental details relating to Chapter 2

8.2.1 Food consumption

The 1997 National Nutrition Survey (Russell *et al.,* 1999) was a key source of food consumption for selected foods. The data collected during this survey include 24-hour dietary recall records for a cross section of 4636 New Zealand residents. These data are the best source of information on the type and quantity of foods consumed in New Zealand. However, the database has limitations, including the fact that it is restricted to people 15 years and older, and food descriptions are not consistently applied throughout the data collection. For instance, a composite food such as 'sandwich' may in some instances be included with no further details, in other instances the same food may be expressed in terms of its component items (bread, margarine, lettuce, salami, etc).

8.2.2 Serum levels of xenoestrogens

An estimate of theoretical serum levels of xenoestrogens corresponding to estimated dietary intakes was made by assuming average serum volumes of 3.3, 2.5 and 2.9l for

males, females and young males respectively based on: blood masses of 7.5% and 7.0% of body weight for men and women; serum volume equates to approximately 55% of blood volume (Lentner, 1984) and; body weights of 80, 65, and 70 kg for New Zealand males, females and young males (Russell *et al.,*1999). It was assumed there was 100% absorption as described by Shaw and McCully (2002) and that all estrogenicity was associated with the serum component of blood. Allowance for factors influencing serum levels was then made by calculating the ratio of actual serum (or plasma) level to actual serum level, based on published data.

Example:

Genistein female intake = 23400 µg/day, measured serum level = 110µg/l
Serum volume =2.5l
theoretical serum level = 23400/2.5 = 9350 µg/l
measured (or actual) : theoretical serum level:
A:T = 110/ 9350 ≈0.01

Serum is plasma minus blood clotting factors and has been used interchangeably with plasma in this thesis.

8.3 Experimental details relating to Chapter 3

8.3.1 Materials

BPA (>99%) and BPA- d $_{16}$ were obtained from Sigma-Aldrich. BPA –d_{16} was converted to PBA-d_{14} by dissolution in aqueous sodium hydroxide and reprecipitation by acidifying with dilute sulphuric acid as described by Goodson *et al.,* (2002). This BPA-d_{14} was used as an internal standard. Acetonitrile, trimethylpentane and acetic anhydride were Analar grade, obtained from BDH.

8.3.2 Extraction

Sample extraction and analysis for BPA was based on the methodology of Goodson *et al*., (2002). All extraction glassware was rinsed with ethanol and acetone before use. The total contents of each can were homogenized in a solvent rinsed, glass Waring blender.

Sample pH was determined on a separate aliquot of the homogenized sample. Duplicate 20.0 ±0.05 g sub-samples of homogenized sample were weighed into 100 ml Schott bottles. One sample was stored at 4°C as a backup. An accurately known quantity of BPA-d $_{14}$ (100µl) was added as an internal standard. The sample was then extracted with 20 ml acetonitrile by shaking on a mechanical shaker for 10-15 mins. For samples containing more than 1% fat (based on label claims), 20ml trimethylpentane (TMP) was also added. This was found to be more effective than heptane as used by Goodson *et al*., (2002). The acetonitrlie portion of the extract was filtered through Whatman No.1 filter paper into a conical flask. The filter cake (and TMP) was re-extracted with a second 20 ml aliquot of acetonitrile. The combined acetonitrile extracts were dried with sodium sulphate (~30g), transferred to a 250ml round bottomed flask, reduced in volume to 5-10 mls by rotary evaporation and diluted to 50 ml with de-ionised water. The extract was derivatised by adding 10ml of 72% w/v potassium carbonate and 10 ml methanol with gentle swirling before the addition of 10 ml acetic anhydride. The solution was left to stand for 15 minutes, swirled, left for another 15 minutes and then transferred to a 100ml separating funnel for extraction with 5 ml heptane. A portion of the heptane layer was transferred to a vial for GCMS analysis. For the analysis of soft drinks, a 50 ml aliquot of degassed sample was derivatised directly.

8.3.3 GCMS analysis

Samples were analyzed on a Shimadzu QP2010 GCMS with an Electron impact analyzer; column, J&W DB5ms, 30m x 0.25mm id, 0.25micron film thickness; carrier gas, zero grade helium at a flow rate of 0.76 ml/min; injector, splitless and temperature programmed 120°(2mins) to 280º (5 mins) at 10ºC/min. BPA diacetyl *m/z* 228, 213; BPA-d_{14} *m/z* 224 ions were monitored. Primary quantitation was measured on the 228 ion, with confirmation on the 213 ion. Standard solutions (5ml) of BPA, including BPA-$d_{14,}$ were derivatised in the same way as sample extracts. Quantitative results were obtained by comparing the ratio of sample response to internal standard response with the ratio of external standard response to internal standard response.

8.4 Experimental details relating to Chapter 4

8.4.1 Materials and Chemicals

Genistein (≥98% pure) and daidzein (≥98% pure) were purchased from Sigma (St Louis, MO). Stock solutions were prepared in HPLC grade methanol at concentrations of 1.49 mmol/L and 1.34 mmol/L respectively. Standards were made for each isoflavone to produce a concentration series from 7 to 120 μmol/L. Brain Heart Infusion (BHI) Broth (Difco) was prepared according to the manufacturer's instructions with water treated by the Elix™ system (Millipore, Molsheim, France). Maxi-Clean C18 cartridges (300 mg, 50 μm particle size, 6 nm pore size) were supplied by Alltech. Anaerobic packets were Oxoid AnaeroGen^{TM} .

8.4.2 Subjects

Informed consent and faecal samples were initially obtained from nine healthy individuals comprising 6 European and 3 Maori New Zealanders. Ethnicity was on a self-claim basis. It was not deemed necessary to consult an ethical committee due to the non-invasive nature of the study. The main subject who participated in all experiments was a European New Zealand female (referred to as Subject A), 47 y of age with a Body-Mass Index (BMI) of 25 kg/m^2. European subjects who participated in further aspects of the study included 1 female (Subject B) and 3 males (C-E), between 31-50 y of age. Four of the participants followed an average omnivorous Western diet with no intentional soy food consumption, the other participant was a vegetarian male who occasionally consumed soy products (Subject E).

8.4.3 Collection and preparation of faecal samples

Fresh, aseptically collected faecal samples were processed within 3 hours. A 5% faecal slurry was made in a whirlpak bag with BHI broth as the diluent, homogenised in a Bagmixer® (Interscience, St Nom, France) for 30 s, and centrifuged at 1660 x *g* for 2 min. Four ml aliquots of faecal slurry supernatant were pipetted into sterile tubes containing the appropriate isoflavone preparation.

8.4.4 Preparation, inoculation and incubation of faecal samples

To measure response at varying isoflavone concentrations (using a sample from Subject A only), three series of tubes were prepared in triplicate containing genistein alone, daidzein alone, and a mixture of genistein and daidzein to final concentrations of 30, 60, 90, 120 and 150 μmol/L of total isoflavone in 5 ml. Controls consisted of tubes containing mixed isoflavones (15 μmol/L of each of genistein and daidzein) in BHI broth (no inoculum), and tubes with faecal supernatant only (4:1 with BHI). All tubes were incubated at 37˚C with anaerobic packets to create micro-aerobic conditions. Where inter-individual variation was studied (all 5 subjects), the faecal preparation method did not change but the final concentrations of isoflavone used in each tube was 59.8 μmol/L genistein, 64.5 μmol/L daidzein, and 124.3 μmol/L total isoflavones in the mixed solutions. Each subject/isoflavone combination was sampled 4 times over 72 h. To assess intra-individual variation with time, the results over 15 weeks from several repetitions of the experiment on Subject A were compared.

8.4.5 Extraction of isoflavones from inoculated faecal samples

Isolation of isoflavones was modelled on the method described in Xu *et al*., (1995). Samples were quickly vortex mixed and 1 ml passed very slowly through a pre-prepared Maxi-Clean (MCC) C18 cartridge. The MCC was ideally pre-wetted with 1 ml 100% methanol, followed by 2 ml water, twice. Once the sample was pushed through the MCC to waste, two rinsing steps followed, each consisting of 2 ml water. The isoflavones were then eluted with 2 ml 80% methanol, reduced in volume by rotary evaporation and made up to 2 ml in volumetric tubes, with 80% methanol, for analysis by thin layer or high pressure liquid chromatography (HPLC).

8.4.6 Thin layer and HPLC protocols

Thin layer chromatography was performed on silica gel plates in a solvent of toluene: ethyl acetate: formic acid (5:4:1). Bands were visualized under UV light (265nm).

HPLC analysis was performed on a Waters WISP 712 coupled with a Waters 600 multisolvent delivery system and Waters Lambda-Max 481 spectrophotometer.

Separation was achieved on a Spheri-5 MPLC ODS (Brownlee; Applied Biosystems, Melbourne, Australia) C-18 column (4.6 x 220 mm, 5 μm particle size) protected by a RP-18 ODS NewGuard (PerkinElmer, Melbourne, Australia) pre-filter column. Methanol/water was used as the mobile phase, employing a gradient of 40% to 65% methanol over 30 min at a flow rate of 1 ml/min. Detection was by UV absorbance at 260 nm. The linearity of response was confirmed for genistein over the concentration range 0-130 μmol/l and for daidzein 0-120μmol/l

8.4.7 Effect of pre-exposure to genistein

In one instance 4 ml faecal supernatant from Subject A was grown in the presence of genistein at a final concentration of 59.8 μmol/l in micro-aerophilic conditions at 37 ˚C (referred to as tube A1). After 30 h, 1 ml was removed and inoculated into a fresh tube of BHI and genistein to give a total of 5 ml at 59.8 μmol/l genistein (tube B). An appropriate amount of genistein was added to the original tube (A1) to achieve a concentration of 59.8 μmol/l (A2). The tubes were then re-incubated and samples analysed over the following 18 h.

8.5 Experimental details relating to Chapter 5

8.5.1 Materials and chemicals

The following reagents, with catalogue numbers in parenthesis, were obtained from Sigma: KH_2PO_4, [P-5655]; $(NH_4)_2SO_4$, [A-2939]; KOH, [P-1767]; $MgSO_4$, [M-2643]; L-leucine, [L-8912]; L-histidine, [H-6034], adenine, [A-2786]; L-argenine.HCl, [A-6969]; L-methionine, [M-2893]; L-tyrosine, [T-8566]; L-isoleucine, [I-7403]; L-lysine.HCl, [L-8662]; L-phenylalanine, [P-5482]; L-glutamic acid, [G-5638]; L-valine, [V-0513]; L-serine, [S-4311]; thiamine.HCl, [T-1270]; pyridoxine, [P-5669]; D-pantothenic acid, [P-2250]; d-biotin, [B-4639]; D-(+)-glucose, [G-7021], L-aspartic acid, [A-4534]; L-threonine, [T-8441]; 17β-estradiol, >98%, [E-8875].

The following reagents, with catalogue numbers in parenthesis, were obtained from Aldrich: $Fe_2(SO_4)_3$, [30,771-8], Inositol, [1 665-2]; $CuSO_4$, [20,917-1]. Chlorophenolred-

β-D galactopyranoside (CPRG), was obtained from Roche, [884 308]. Nunc [260860], 96-well flat-bottom microplates were obtained from Invitrogen.

8.5.2 Preparation and storage of minimal medium and medium components

Minimal medium, vitamin, D-(+)-glucose, aspartic acid, threonine, copper sulphate, growth medium and CPRG solutions were prepared according to the following protocol supplied by Beresford and Sumpter (personal communication, 2002) with minor modifications as indicated.

Minimal medium and medium components prepared in glassware contaminated with an estrogenic chemical will lead to elevated background expression. Glassware, spatulas, stirring bars, etc., were scrupulously cleaned, and not had prior contact with steroids. Glassware, spatulas, stirring bars were rinsed twice with absolute ethanol, and left to dry.

Minimal Medium (PH 7.1)

13.61 g KH_2PO_4, 1.98g $(NH_4)_2SO_4$, 4.2g KOH pellets, 0.2 g $MgSO_4$, 1 ml $Fe_2(SO_4)_3$ solution (40mg/50 ml H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine were added to 1 L double-distilled water and placed on heated stirrer to dissolve. Aliquots (45 ml) were dispensed into glass bottles,sterilised at 121^{o}C for 10 min, and stored at room temperature (up to 3 months).

D-(+)-Glucose

A 20% w/v solution was prepared, sterilised in 20 ml aliquots at 121^{o}C for 10 min. and stored at room temperature.

L-Aspartic Acid

A stock solution of 4 mg/ml was prepared, sterilised in 20 ml aliquots at 121^{o}C for 10 min and stored at room temperature.

Vitamin Solution

8 mg thiamine, 8 mg pyridoxine, 8 mg panthothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml H_2O) were added to 180 ml double-distilled water. The solution was sterilised by filtering through a 0.2-µm pore size disposable filter, in a laminar air flow cabinet, filtered into sterile glass bottles in 10 ml aliquots and
stored at 4^{o}C.

L-Threonine

A solution of 24 mg/ml was prepared, sterilised in 10 ml aliquots at 121^{o}C for 10 min and stored at 4^{o}C.

Copper (II) Sulfate

A 20mM solution was prepared and sterilised by filtering through a 0.2-µm pore size filter, in a laminar flow cabinet. The solution was filtered into sterile glass bottles in 5 ml aliquots and stored at room temperature.

Cholorophenol red-βD-galactopyranoside (CPRG)

A stock solution of 10 mg/ml was prepared, sterilised by filtering through a 0.2-µm pore size filter into sterile glass bottles, in a laminar flow cabinet and stored at 4^{o}C.

8.5.3 Preparation and storage of yeast stocks

Yeast was prepared and stored according to the following protocol supplied by Beresford and Sumpter (personal communication, 2002) with minor modifications as indicated.
All yeast work was carried out in a type II laminar flow cabinet.

8.5.3.1 Short term storage (-20°C) of 10x concentrated yeast stock culture

Day 1

Growth medium was prepared by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin, 0.4 ml L-threonine solution, and 125 µl copper (II) sulphate solution to 45 ml minimal medium. The solution was transfered to a sterile conical flask

(final volume of approximately 50 ml) and seeded with a 125 µl aliquot of 10X concentrated yeast stock from a cryogenic vial stored at –20°C. This was incubated at 28°C for approximately 24 hour on an orbital shaker, or until turbid. (Yeast from a cryovial stored at –80°C was also be used.

Day 2

Growth medium was transfered to two conical flasks (each with a final volume of approximately 50 ml). Yeast (1ml) from the 24-h culture was added to each flask and incubated at 28°C for approximately 24 hour on an orbital shaker, or until turbid.

Day 3

Each 24-h culture was transferred to a sterile 50-ml centrifuge tube. Cultures were centrifuged at room temperature (not 4°C as specified) for 10 min at 2,000 g. The supernatant was decanted, and each culture resuspended in 5 ml of minimal medium with 15% glycerol (add 8 ml sterile glycerol to 45 ml minimal medium). Aliquots (5ml) of the 10X concentrated stock culture was transferred to 1.2-ml sterile cryovials and stored at –20°C for a maximum of 4 months.

8.5.4 Assay procedure

All preparations of assay solutions and assay plates were carried out in a laminar flow cabinet to minimize microbiological contamination of the yeast cultures.

Day 0

Growth medium was prepared and seeded with yeast in the same way as for the -20°C yeast storage (8.5.3.1).

Day 1

100µl of test chemical solutions of the appropriate concentrations in ethanol were pipetted into the first column of a selected row of a 96 well plate. 50µl ethanol was pipetted into all other wells of the row. The test chemical was serially diluted across the row by pipetting 50µl aliquots into successive wells. 10µl of each concentration was transferred to a second row, and allowed to evaporate. 17β-Estradiol (over the concentration range

0.0049 to 10nM per well) was used as a positive control, and ethanol used as a negative (or blank).

For each assay, a fresh bottle of growth solution (50ml) was seeded with 250µl of a 24h yeast culture (optical density at 640nm of 2.5), from Day 0 and 0.5 ml CPRG. 200µl aliquots of this assay medium was added to each well, the plates were sealed with autoclave tape and shaken in a fixed wavelength plate reader (BIO-TEK EL312) for 2 minutes. The plates were incubated for about 48h at 30°C, until a colour degradation from red to yellow was observed across the estradiol standard. The red colour denoting estrogenicity was measured at 540nm and 610nm. 610nm measures the turbidity of the medium, hence the growth rate of the yeast. The Beresford/Sumpter protocol stated measurement of turbidity at 620nm, however, we measured turbidity at 610nm because of the availability of this fixed wavelength filter. The negative controls appeared a pale orange colour due to background β-galactosidase activity. The absorbance at 540nm was therefore corrected by applying; A_{540}-(A_{610}-blankA_{610}).

$$\text{Corrected value} = A_{540} - (A_{610} - \text{blank } A_{610}).$$

Concentrations of the test chemicals, 17-β estradiol, bisphenol A and genistein were adjusted until a 'S' curve was achieved on analysis with SigmaPlot 2000: estradiol (54.48 µg/l), bisphenol A (349 mg/l) and genistein (278 mg/l). Absorbance values were entered into an excel spreadsheet for the calculation of 'corrected values' that were presented graphically using SigmaPlot. An example spreadsheet is shown in Appendix 3. EC_{50} values, the concentration of test chemical required to induce 50% of maximum estrogenic effect, were calculated from the line fit generated in SigmaPlot.

8.6 Experimental details relating to Chapter 6

Perfusions were undertaken by Mi-kyung Iris Shin, University of Auckland as part of a Master's thesis (Shin, 2004).

8.6.1 Tissue collection

Placentas were obtained from healthy women undergoing elective caesarean section at term at the National Women's Hospital, Auckland, New Zealand. Samples were collected between July 2003 and May 2004 with informed consent and ethics approval. Placentas were obtained within 5 min of delivery and transported immediately to the laboratory.

8.6.2 Dual perfusion technique

The perfusion procedure was based on the method of Cannell *et al.,* 1988. Perfusates were prepared from tissue culture medium 199 (Irvine Scientific California), with added sodium bicarbonate pH 7.4 containing 5% polyvinylpyrolidone 40 (BDH laboratories), 0.1% bovine serum albumin (Sigma), 48μg/ml gentamicin (Sigma) and 20 IU/ml heparin (Sigma). The medium was gassed with 95% oxygen and 5% carbon dioxide (BOC gases, Auckland) in a 37˚ C water bath in the chamber. The perfusate was freshly prepared for each perfusion. Stock solutions of 17β-estradiol and genistein (Sigma Aldrich) were prepared in ethanol (99%) and stored at -20˚.

Dual perfusions were undertaken in a 5% humidified compartment at 37˚ C by selecting a suitable lobule and cannulating a fetal vein and artery with 18 gauge infusion needles within 10 minutes of delivery. Once fetal arterial flow was started (1 ml/min), the corresponding cotyledon on the maternal side was identified and perfused by placing butterfly needles into the appropriate intervillous space 0.5cm below the maternal surface. Perfusate volumes of 100 and 200 ml were used on the fetal and maternal sides respectively. Flow rates on the fetal and maternal sides were adjusted 3 and 10 ml/min respectively, whilst maintaining fetal arterial pressure at less than 40mmHg. The system was equilibrated for 20 mins. Tygon tubing 1/8 inch (i.d) in the maternal circuit and 1/16 inch (i.d) in the fetal circuit was used for the perfusion circuits. Pressure in both circuits was monitored with an aneroid sphygmomanometer connected to the fetal and maternal artery respectively. After equilibration, the maternal and fetal reservoirs were changed for fresh perfusates. Test compounds were included in the maternal perfusate only. Samples

were taken for metabolic viability and estrogenicity or concentration analysis at fixed intervals (usually hourly) using a disposable plastic syringe.

8.6.3 Viability of perfusions

Physical markers of integrity and biochemical markers of metabolic viability were undertaken as described by Cannell *et al*., (1986, 1988). Physical parameters included placental weight, fetal arterial pressure and leakage. Oxygen and carbon dioxide saturation, electrolytes (sodium and potassium), glucose consumption, lactate production and human chorionic ganadotrophin (hCG) analyses were undertaken by the chemical pathology laboratory at National Women's Hospital.

8.6.4 Estrogenicity in maternal and fetal perfusates

Blank perfusions and perfusions of 17β-estradiol (1μg/l) were measured for estrogenicity, as received, using the yeast assay as described in Chapter 8.5. A comparison of estrogenicity between maternal and fetal samples at equivalent sampling times was made from respective absorbance readings at 540 nm corrected for difference in turbidity (as a measure of cell growth) by subtracting the value for absorbance at 610nm. (i.e. Abs 540-Abs 610).

8.6.5 Analysis of genistein in perfusates.

Genistein was extracted from fetal and maternal perfusate samples by methodology developed for the gut microfloral work described in Chapter 8.4.5. In this instance, a maxi-clean C-18 cartridge was pre-wetted with 3ml of methanol (99.9%, Mallinckrodt) followed by 3 ml water. Perfusate solution (1 ml) was dripped through the cartridge followed by 3 ml water. Genistein was eluted with 80% methanol in water (1 ml) and analyzed by HPLC.

HPLC analysis was performed on a Shimadzu SCL-10A liquid chromatograph coupled with a Shimadzu SPD-M10A diode array detector. Separation was achieved on an Applied Biosystems RP-18 (Brownlee; Applied Biosystems, Melbourne, Australia) C-18 column (4.6 x 100 mm) protected by a RP-18 (3.2 x 15 mm) guard column (Brownlee;

Applied Biosystems, Melbourne, Australia). Methanol/water (50:50) was used as the mobile phase at a flow rate of 1.5 ml/min. Detection was by UV absorbance at 260 nm.

8.6.6 Recovery of genistein from spiked perfusates solution

A blank perfusate solution, spiked with genistein at a concentration of 220µg/l was analysed with each HPLC run. Recovery of genistein was 86.4 and 95.5% for the two analytical runs. The results were not adjusted for recovery of genistein.

8.7 Experimental details relating to Chapter 7

8.7.1 Materials and chemicals

RPMI media was Gibco, available from Invitrogen (Ref. 31870-025). Genistein, bisphenol A, TCA (Trichloroacetic acid,T6399) and sulfohodamine (S1402-5G) were obtained from Sigma.. Tris(hydroxymethyl)methylamine was BDH (103156X).

8.7.2 Cell lines and cell culture

Human cancer cell lines including prostate (DU 145), testicular (SuSa and 833K) colon (HT29) and breast (MCF-7) were kindly supplied by Professor John Masters, Director, Cancer Research Laboratory, University College, London. All these cell lines are adherent. Cell culture media was prepared by adding 40mls Foetal Bovine Serum and 5ml L-Glutamine to 500mls RPMI 1640 media. Cells were maintained in 5ml media in T25 flasks, incubated at 32°C.

8.7.3 Measurement of cell growth curves

Growth media was aspirated from the T25 incubation flask of growing cells, the cells released with trypsin (1ml) and resuspended in 10 fresh media. A 0.2ml sample of this culture was stained with trpan blue (0.2 ml of 1% solution) and cells were counted on a haemocytometer. Varying amounts of this culture was diluted to 10ml with media to give

cell counts ranging from 0-15,000 cells per 200µl. Up to 12 different dilutions were prepared for each cell line. Eight replicate aliquots (200µl) of each dilution was added to one column of a 96 well plate (12 columns). Five plates were prepared for each cell line. The plates were incubated at 32°C. cell growth was measured each day, for 5 days, using the SRB assay.

8.7.4 Dose response of cells to estrogenic compounds

Standard solutions of the endogenous hormone 17β- estradiol, the naturally occurring xenoestrogen genistein and the synthetic xenoestrogen bisphenol A were prepared in ethanol (1.9 x 10^{-3} M, 1.5 x 10^{-3} M and 2.4 x 10^{-3} M respectively) and serially diluted over the concentration range of 10^{-4} to 10^{-11} M. Aliquots (20 µL) of individual standards were added to Nunc flat bottomed 96 well plates (1 plate per compound per cell line) and left to evaporate before the addition of 200 µL of sufficient cell culture medium to achieve exponential growth at day 3 (72 hrs). This time would allow for multiple cell cycles to mimic chronic exposure as in the human situation. From the previous growth curve data, a cell density of 7000 cells/200µL was selected as optimal. Cells were incubated at 32°C for 3 days. Cell growth was determined by the SRB assay.

8.7.5 Sulfohodamine (SRB) assay

This assay developed by Skehan *et al*., (1990) measures cell growth as cell protein, visualized by binding to the dye SRB. 100µl of a 30% (w/v) aqueous solution of trichloroacetic acid (TCA) was added to each well in a 96 well plate and allowed to fix for at least 20 mins. Flicking and blotting onto paper removed the TCA. Cells were washed carefully 4-5 times with tap water. 100µl of a 0.4% solution of SRB (in 1% acetic acid) was added to each well and allowed to stain for at least 20 mins. The stain was removed by flicking and the cells washed 5 times with 1% acetic acid. Cells were left to dry overnight in a fume hood. The stain was then solubilised with 100µl 10mM tris(hydroxmethyl)methylamine base for 20 mins. Plates were tapped to mix and absorbance measured at 540nm on a Perkin Elmer Wallac Victor 1420 multilabel counter with "Workout" software. Absorbance was linear up to a value of 2.0.

8.7.6 Exposure of cells and transport to New Zealand.

8.7.6.1 Exposure of cells to estrogenic compounds

On the basis of dose response results, cells were exposed at concentrations approximating serum levels. The following volumes of standard solutions diluted in ethanol were added to T80 flasks. Flasks were left open in laminar flow cabinet until the ethanol had evapourated. The cells of a T25 flask were released with trypsin, media was added (15ml) and the contents transferred to the T80 flasks. For HT29, DU145 and SuSa the T25s were confluent before transfer to the T80. For 833K and MCF-7 the t25s were about 50% confluent. Flasks were incubated at 32°C for 3 days.

Table 8.7.1: Level of exposure of HT29, DU145, SuSa, MCF-7 and 833K cell lines to 17β-estradiol, genistein , BPA and genistein + BPA.

	Conc (M)	**Dilution factor**	**Volume added (ml)**	**Volume of media (ml)**	**Final conc. (M)**
Estradiol	1.86×10^{-3}	10^{-5}	1.5	15	1.8×10^{-9}
Genistein	1.54×10^{-3}	2×10^{-3}	0.6	15	3.1×10^{-6}
BPA	2.42×10^{-3}	10^{-3}	0.75	15	1.2×10^{-7}
Genistein + BPA	1.54×10^{-3}	2×10^{-3}	0.6	15	3.1×10^{-6}
	2.42×10^{-3}	10^{-3}	0.75	15	1.2×10^{-7}
Control	0	0	0	15	0

8.7.6.2 Preparation of cells for transportation

Flasks were examined under the microscope. Cells in four of the 20 exposure scenarios showed fungal contamination and were abandoned. Media from the remaining flasks was aspirated off and the flasks washed with 3 x 3ml fresh media (without FBS or L-Glutamine). Cells were scraped from the flasks (to minimize lysing of cell walls), transferred to a centrifuge tube with media (7 ml) and centrifuged (1000rpm , 5 min.). The supernatant was discarded and the plug resuspended in media (1ml). Aliquots (0.5ml) were transferred to 2 cryovials, centrifuged (1000rpm, 5 min), the media discarded and the cryovials snap-frozen in liquid nitrogen. Vials were labeled and stored at -80°C until dispatch to New Zealand on dry ice and subsequent storage at -80°C until analysis.

Chapter 9

Overall discussion on dietary xenoestrogens.

The question is, could xenoestrogens from the diet affect human health? Are they likely to have a long-term effect at a population level? The new information generated in this thesis will be considered in light of the published literature in the hope of answering these questions.

International interest in EDCs, of which xenoestrogens are a major subset, continues, albeit less in the lime-light of the popular press. Whilst effects on wildlife are apparent the effects of low-level exposure and adverse human health effects remain suggestive but not proven. Current concern at a government level over the possible effects of EDCs is however, reflected in the millions of dollars allocated by European and American agencies to support research on EDCs, for example $23 M by the European Union in 2003 (Lorenz, 2003). Clearly governments are concerned about the effects of these chemicals on their populations. This research is wide ranging and includes, for example, food safety issues of phytoestrogens, the screening of many thousands of chemicals for endocrine effects, how these chemicals interact with one another and how they influence gene expression. It is likely that these large government-funded programmes will shed significant light on the issue.

Xenoestrogens are foreign to the body but mimic the action of estrogens, hormones that promote the development and maintenance of female sex characteristics. Whilst recognizing that there are various mechanisms by which environmental compounds might influence the level and balance of sex hormones in the body, this thesis focuses on dietary xenoestrogens that are known to bind and activate ER via the classic genomic pathway of

ligand-dependent activation of the estrogen receptors (ERα and ERβ) leading to a conformational change in DNA, transcription to mRNA and altered expression of specific genes (Gruber *et al.,* 2002), as a basis for the selection of xenoestrogens from food, so that potential additive effects can be discussed. A common mechanism of action is a requirement for additivity of effect from exposure to a mixture of chemicals (Rudel, 1997, van den Berg, 2000).

Many compounds with *in vitro* estrogenic activity have been detected in a wide range of foods. Hence all humans will be exposed to xenoestrogens through their diet. Naturally occurring xenoestrogens include the isoflavones (genistein and daidzein), the lignan metabolites (enterolactone and enterodiol), coumestrol, and the flavonoids (apigenin, isoliquiritigenin, kaempferol, luteolin, naringenin, phloretin and quercetin). Synthetic compounds that have been shown to have estrogenic activity, and have been found in foods, include pesticides (DDT and metabolites, dieldrin, endosulphan and synthetic pyrethroids) and industrial chemicals (PCBs, alkyl phenols, BPA and BHA). Although public perception may perceive synthetic chemicals to be more risky than natural chemicals, the real risk of the synthetic chemicals can only be determined in context of the total risk of xenoestrogens from food.

The extensive collation undertaken in this thesis, of existing data on concentrations of these compounds in food, consumption information for each food for different population groups, reported serum levels and relative estrogenicity information from the literature underpins the most comprehensive exposure assessment and ranking currently available in the international literature (Chapter 2). Risk, expressed as estrogenicity relative to normal circulating levels of endogenous estrogens, has been calculated for this level of exposure for four population groups, with discussion around an additional group, pubescent boys. Drawing extensively on pre-existing data, the current assessment has created new knowledge of data gaps, a more comprehensive ranking of contributing xenoestrogens and an estimate of the total level of risk from exposure to estrogenic compounds across the diet, taking into account, factors influencing human serum levels.

Any risk assessment has attendant uncertainties (Covell and Merkhofer, 1996) and the uncertainties and biases for even a small number of aggregated chemical components can

be substantial (USEPA, 2000). In the present example uncertainties relate to each component of the assessment, namely the concentration data, food consumption information, estrogenic potency and serum data. In each case, the risk assessment has been derived from hard (measured) data. However, data for some xenoestrogens are more robust (less uncertain) than for other xenoestrogens. A qualitative assignment of the robustness of data for one xenoestrogen compared with another indicates that the least robust data in the risk assessment is due to the lack of appropriate serum information for the sub-populations under study, including data on serum levels for coumestrol, luteolin, apigenin, isoliquiritigenin, zearalenone, endosulfan, synthetic pyrethroids and alkyl phenols. In the absence of more appropriate data, extrapolations from one population to another were assumed to accommodate these data gaps. This may be erroneous. These compounds could have been excluded from the assessment, however this would have presented a very incomplete picture of major contributors and major data gaps.

The risk assessment shows that estrogenicity from the diet is coming primarily from the naturally occurring isoflavones (genistein and daidzein) and flavonoids (particularly isoliquiritigenin), the contaminant mycotoxin, zearalenone, with minor contributions from nonyl phenol, a pesticide excipient and industrial surfactant, and the synthetic compound BPA. The relative contribution of each xenoestrogen is not only a function of potency relative to estradiol (Sharpe and Skakkebaek, 2003, vom Saal and Hughes, 2005) but also of intake and those factors that influence the A:T ratio, namely absorption, metabolism and excretion. Hence genistein and zearalenone contribute similarly despite the 10,000 times difference in intake. The contribution from estrogenic pesticides and PCBs is negligible because of low levels of exposure combined with low potency, yet these chemicals have been the targets of numerous epidemiological studies of potential impacts of EDCs on human health (Alavanja *et al.,* 2003, Bhatia *et al*., 2005, Buck *et al.,* 1999, Cooper *et al.,* 2005, Fuortes *et al.,* 1997, Hoyer *et al.,* 1998, ICPS, 2002, , Rignell-Hydbom *et al.,* 2005, Settimi *et al.,* 2003 Sturgeon *et al.,* 1998, and Windham *et al.,* 2005). Some of these studies were very reasonably targeting historical exposure (Bhatia *et al*., 2005, Cooper *et al.,* 2005, Krstevska-Konstantinova *et al.,* 2001) but the results presented in this thesis suggest that future studies might consider the inclusion of isoflavones, isoliquiritigenin, zearalenone, nonyl phenol and BPA as potential causative agents for estrogenic effects.

Exposure to the naturally occurring isoflavones, genistein and daidzein for omnivorous adults comes largely from foods containing soy ingredients, such as bread and processed meats, and for males, from beer. For a vegetarian female, 80-90% of isoflavone intake is attributable to genistein from the consumption of soy milk. Soy production in the USA has increased 48% between 1971 and 1996 (Shaw, 2004) and some of this will be going into the human diet. The increasing use of soy as a component of processed foods such as prepared meats and bread, means that exposure to estrogenic compounds in the diet, hence potential risk, will increase in the future. The safety of this increasing use of soy as a food ingredient has been the topic of press articles last year, both in New Zealand (Allan 2004) and the United Kingdom (Barnett, 2004) and warrants further study. Once again, this is a controversial area because Asian people consume much higher levels of soy than those on a Western diet (Table 2.2.4, Uehar *et al*., 2000) with no apparent disadvantage, and with lower rates of the "hormonal" breast and prostate cancers (IARC, 2004).

Any estrogenic risk from the naturally occurring phytoestrogens (including both isoflavones and flavonoids) needs to be considered in context with the claimed benefits of these compounds whether that benefit is via an estrogenic or non-estrogenic mechanism. The safety of dietary supplements and food additives containing phytoestrogen extracts does warrant concern because the risks may be concentrated and the benefits of the whole foods from which they are derived, compromised. Professor Helferich's group at the University of Illinois will hopefully shed light on this topic (IFT, 2004).

The contribution of zearalenone to total estrogenicity is due to its high estrogenic potency relative to other xenoestrogens and an occurrence of about 7% in corn based foods. The estrogenicity of zearalenone has been confirmed by 5 authors (Hobson *et al.,* 1977, IEH 2000, Kiang *et al.,* 1978, Soto *et al*.,1995, Welshons *et al*., 1990) using either receptor binding, cell proliferation and *in vitro* assays and is therefore robust. Currently there is a marked paucity of data on human serum levels of zearalenone and no information of serum levels relative to dietary intake. One report (Pillay *et al.,* 2002) of serum levels of zearalenone for 132 South African female subjects is 1000 times higher than might be estimated for a Western type dietary intake. Given the relatively high estrogenic potency of zearalenone, one would expect that if the serum level found in the South African women is also found in post-menopausal women and/or African men, then a human health effect would be apparent in these population groups. In the absence of more data, the

current estimate assumes all ingested zearalenone is bioavailable (but not accumulated). Given the contribution of zearalenone to total estrogenicity, clearly more information on dietary exposure, serum levels and metabolic pathways of this toxin is a priority area for research.

The industrial surfactant or pesticide excipient, nonyl-phenol, is estimated to contribute about 5% to total estrogenicity in the current assessment, although there is a high degree of uncertainty associated with this estimate. The concentration data was for German foods (Guenther *et al.,* 2002) and most likely does not reflect use in the country of consumption (New Zealand). In addition the serum level used to derive the A:T ratio was based on data for Malaysian women (Tan and Mohd, 2003). Nonyl phenol is used in some pesticide preparations in New Zealand (Alan Cliffe, Nufarm, personal communication, October 2002) but the extent and frequency of use, and discharge into the New Zealand environment is not known and is a priority data gap. So too is the wider issue of including the potential effect of "non-active" agents that are used in industrial preparations. In the EU, the use of nonyl phenol is restricted in order to protect the environment (EU, 2003). Evidence from the work in this thesis supports such initiatives.

The synthetic estrogenic compound, BPA, is estimated to contribute about 3% to the total daily intake of estrogenicity from the diet. This estimate is based on a comprehensive survey of BPA, in canned foods available to the New Zealand consumer (Chapter 3). Although levels of BPA were generally low, there were a few foods with sporadically high levels that contributed to exposure. The foods with sometimes elevated concentration of BPA were imported samples of tuna, corned beef and coconut cream from Thailand (tuna and coconut cream) and Australia. The reason for the elevated levels of BPA may be related to fat content, because BPA is fat soluble, but is more likely to be a factor of processing (Goodson *et al.,* 2004). The exposure estimates using concentration information generated from this study with consumption information from the New Zealand National Nutrition Survey (1997) has provided a robust exposure assessment (Thomson and Grounds, 2005). Since the mean and maximum exposures (0.008 and 0.29µg/kg bw/day respectively), are below the current temporary Tolerable Daily Intake of 10 µg/kg bw/day given by the European Commission in 2002 it would appear that the levels of BPA identified in canned foods are unlikely to be of concern to adult health and there is no reason for consumers to change their consumption patterns as a result of these

findings. The international guideline is based on a three-generational rat study (SCF, 2002) where reductions in body and organ weights were observed following exposure to BPA. The questionable relevance of these endpoints for human health is one of the reasons that the TDI remains temporary. This TDI also considers exposure to BPA alone and not as one of several compounds contributing to total estrogenicity. It will be important to revise the temporary TDI for BPA as further information is available, as most recently recommended by vom Saal and Hughes (2005).

The daily intake of BPA for a New Zealand woman of 0-17.9, mean 0.46, µg/day (Chapter 3) spans the range of intakes for Japanese pregnant women of <0.3-7.9 µg/day reported by Fujimaki and colleagues (2004). For the same sub-population (but different individuals) Takeuchi *et al.,* (2004) reported higher serum levels of BPA (1.05 ng/ml compared 0.71 ng/ml, $p<0.05$) for normal weight women with ovarian disease (polycystic ovary syndrome) compared with a healthy control group. Assuming equivalent dietary intakes between the two groups of Japanese women, the level of exposure of New Zealand women to BPA is approximately equivalent to the subjects in the Japanese study and may be associated with polycystic ovary syndrome. Because the difference in BPA serum levels between normal and subjects with polycystic ovary syndrome was not observed for obese women (Takeuchi *et al.,* (2004), it is important that the association is substantiated by independent confirmation.

There is evidence of additivity of effect of low doses of xenoestrogens based on experiments using the yeast assay, (Payne *et al.,* 2000, Silva *et al.*, 2002, Rajapakse et al., 2002) cell proliferation (Rajapakse *et al*., 2004) and gene expression assays (Gaido *et al.,* (2003). However, additivity of xenoestrogens is neither guaranteed nor predictable because the effect in combination may be different to the effect of the compound when present as a single compound (Gaido *et al.,* 2003, Rajapakse *et al*., 2004) and because the effect may vary in different target cells (Gaido *et al.,* 2003). Each of the combinations cited above uses a different selection of xenoestrogens and each system is *in vitro,* not accounting for endocrine effects of a complete organ let alone a whole animal. The risk assessment (Chapter 2) ranks the estrogenic contribution of dietary xenoestrogens for a Western diet providing a sound basis for selecting which compounds, in what proportions, to combine in future additivity studies.

If the xenoestrogen effect is additive, the conservative, precautionary approach taken in this study suggests that combined xenoestrogens from the diet, when compared with endogenous levels of estradiol, might almost double the circulating levels of estrogenicity for all males and for post menopausal women consuming an average diet. This is not an insignificant increase and could plausibly have a pharmacological effect. For a reproductive female it is hard to see how an extra 3% of estrogenicity from the diet could have an effect because a woman's body is physiologically adapted to major cyclic fluctuations of estrogen levels during the menstrual cycle. The contribution of estrogenicity from food is small in comparison to the amount of naturally circulating estrogen during the month. But at menopause this changes as she becomes hormonally more male and estrogen levels are much reduced (Greenspan and Gardner, 2001). Therefore the dose at menopause may be pharmacologically significant.

Perhaps the most at-risk group is pre-pubescent males, because changes in circulating estrogen activity might change the course of their sexual development. Clearly estrogen plays a key role in the growth associated with puberty (MacGillvray, 2004). There are no New Zealand food consumption data for this group, and therefore it was not possible to include them in our model. However, Australian data (ABS, 1999) indicates the pre-pubescent males (8-11 years) eat a similar range of food to adult males (25-44 years) and in similar quantity (10% less across the diet). Their much lower body weights (mean 34.6 kg against a mean of 82.4 kg for an adult male) mean that their xenoestrogen dose on an intake per body weight basis would be approximately twice that for adult males (i.e. 12-16 ng/l). Whether this additional estrogenicity from exogenous sources disturbs pubertal development remains plausible but speculative (Tielmann *et al.,* 2002).

Serum levels of the naturally occurring isoflavones genistein and daidzein are much lower than estimated from dietary intake (Chapter 2.2.4). Gut microflora clearly have a role in the bioavailability, hence dose, of these phytoestogens (Chapter 4). Some microflora can metabolise the conjugated isoflavone to the less polar aglycone but the identity of the organisms and the mechanism of metabolism of the aglycone is not known. Although it is preferable to undertake degradation studies with microflora from the small intestine, where the majority of absorption is likely to occur, this was not achievable. Therefore gut microflora were sourced from human faecal material to study the microbial degradation of genistein and daidzein, a pragmatic approach also taken by other investigators (Xu *et*

*al.,*1995, Setchell *et al*., 1984). The variability in the ability of faecal gut microflora to degrade genistein and daidzein, and hence influence available dose, applies to both claimed benefits and possible risks from dietary exposure to these phytoestrogens. Within and between individual variability is particularly interesting in the context of the promotion of the consumption of soy as a functional food. On the basis of experimental work undertaken as part of this thesis, the bioavailability of dietary sources of isoflavones is highly unpredictable and therefore the dose to target organs must be highly variable, and so too the beneficial effect. How can health claims be substantiated?

In utero exposure to xenoestrogens has been implicated in neurobehavioural and testicular dysgenesis syndrome (including cryptorchidism, hypospadias, testis cancer and low sperm count) effects (Sharpe and Skakkebaek, 1993, 2003, Shirai and Asamoto, 2003, O'Connor and Chapin, 2003). Implicit in an *in utero* effect is transfer from the mother to the fetus. The risk assessment undertaken within this thesis shows that assuming additivity, the combined contribution of estrogenicity from dietary xenoestrogens is very low, (0.02-0.2%) compared with the very high levels of endogenous estradiol during pregnancy (10-30,000 ng/l, Beard and Nathanielsz, 1984). This substantiates the view that it seems unlikely that altered human exposure to weak estrogenic compounds can account for the possible increasing incidence of male reproductive tract disorders (Sharpe and Skakkebaek 2003).

However, three generational effects of exposure to the estrogenic drug DES (Klip *et al*., 2002), causal associations between exposure and health risks from epidemiological studies (Weidner *et al*., 1998, Perera *et al.,* 2003), combined with evidence for the placental transfer of both naturally occurring (Adlercreutz *et al.,* 1999) and synthetic xenoestrogens (Miyakoda *et al.,* 1999, Uchida *et al.,* 2002) from animal studies, substantiate the potential for effects from *in utero* exposure to xenoestrogens.

Despite limitations, the *ex vivo* dual perfusion placental model is a challenging but safe, ethically acceptable, appropriate technique for modelling the placental transfer of xenobiotics in humans (Ala-Kokko *et al.,* 2000). Evidence from the studies presented (Chapter 6) shows that the placenta limits the transfer of estradiol from maternal to fetal compartments and suggest that about 10% of the naturally occurring phytoestrogen, genistein, transfers from the maternal to fetal compartments when perfused as an individual chemical in a human placenta. In comparison with some drug transfers

(Myllynen and Vähäkangas, 2003, Rama Sastry *et al*., 1998) this result demonstrates that the placenta has a limiting effect on placental transfer. Subsequent studies with a synthetic xenoestrogen and xenoestrogens in combination with endogenous 17β-estradiol will help to resolve the discriminatory function of the placenta, hence transfer of xenoestrogens from mother to fetus and fetal "dose".

North and colleagues (2000) suggested the increased incidence of hypospadias in sons of women consuming vegetarian diets during pregnancy might be attributable to increased exposure to phytoestrogens for the vegetarian mothers. The present risk assessment shows that vegetarian females are indeed exposed to a higher level of estrogenicity from the diet than omnivorous females by a factor of approximately two. Most of this difference is attributable to increased exposure to zearalenone from a higher consumption of mixed grain bread by the vegetarian mothers. Whether this is of any significance to fetal development is simply not known.

Research on *in utero* exposure supports the debate on "You are what your mother ate", that your health as an adult may be determined to some extent by imprinting that occurs from maternal exposure. The impact of this exposure on the developing fetus surely warrants further study, especially at the critical periods of sexual differentiation and development of the endocrine system (around 8-12 weeks gestation). Such research aligns with the international prioritization of the identification of the life stages that are most vulnerable to the effects of endocrine disrupting compounds.

The final component of this thesis introduces the concept of a genomic approach to explore the mechanistic link between exposure to xenoestrogens and human health effect. This "is" the future. Emerging "omics" technologies (genomics, proteomics, metabolomics, toxicogenomics) offer exciting opportunities to substantiate cause and effect, at a biochemical level, of hazards that are mitigated by altering gene expressions such as the classical pathway of estrogens. In this way it will be possible to identify key candidate genes (and proteins) that are up or down regulated by which dietary xenoestrogens in which target tissues. When these are known, susceptibility of individuals to exposure to xenoestrogens will be predictable on the basis of whether the individual expresses the relevant genes, i.e. the person's genotype. An example of progress in this area is the work achieved by Professor Wittliff, University of Louisville (Personal communication, March 2004), who has identified 200 estrogen responsive

genes from breast cancer patients and the grouping of breast cancer patients based on these gene expressions. It is a logical step to determine how, or indeed if, the expression of these genes is altered by exposure to xenoestrogens. From the risk assessment, Chapter 2, health effects on males are considered a priority area and prostate cancer, male infertility and sexual differentiation *in utero* are target health outcomes for genomic methodologies.

In addition to classical genomic actions, estrogens can act through non-genomic or membrane-initiated signaling pathways via a membrane form of ER (mER). Xenoestrogen actions via nongenomic pathways are largely unstudied but in a recent publication Wozniak *et al.,* (2005) report on *in vitro* studies with a pituitary tumor cell line, selected for expression of high levels of membrane ER-α. Low concentrations of estradiol, BPA, coumestrol, DDE, DES, dieldrin, endosulfan and nonylphenol (10^{-8} to 10^{-12} M) mediated membrane initiated intracellular Ca^{++} increases resulting in secretion of prolactin. No Ca^{++} changes occurred in cells deficient in mER-α, demonstrating a ER mechanism. Overstimulation, inappropriate stimulation (for developmental stage or reproductive cycle stage), or inhibition of prolactin secretion can lead to a variety of disruptions of normal reproductive function (Wozniak *et al.,* 2005). The serum levels derived for a young male for BPA, coumestrol, DDE, endosulfan and nonyl phenol (Chapter 2) are 10^{-6}, 10^{-7}, 10^{-6}, 10^{-8} to $10^{-9}$and 10^{-5} M respectively, well above the concentration at which the greatest changes in intracellular Ca^{++} were observed.

Whilst there are yet no definitive causal associations between exposure to xenoestrogens in the diet and human health, there clearly remains evidence for concern. Further exciting research awaits on:

- the role of gut microflora on the bioavailability of phytoestrogens and how this may be influenced by diet
- quantification and the significance of *in utero* exposure to both naturally occurring and synthetic xenoestrogens at dietary levels
- the additivity of xenoestrogens using genomic technologies and combinations at environmental levels, derived from this work
- exploration of genomic effects of dietary xenoestrogens in selected tissues, in the first instance targeting male health issues

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Appendices

Appendix 1: Intake estimates

Estimated intake of apigenin

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
celery	108	2.9	2.9	5	2.9	0.313	0.313	0.540	0.313	100	100	100	100

Estimated intake of coumestrol-upper

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Alfalfa sprouts1	46.8	0.47	0.19	0.36	0.36	0.022	0.009	0.017	0.017	67.6	45.8	61.6	61.6
Alfalfa tablets2	1285					0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
Alfalfa and kelp2	847	0.01	0.01	0.01	0.01	0.008	0.008	0.008	0.008	26.0	43.6	30.9	30.9
kala chana seeds1,	61.3	0.01	0.01	0.01	0.01	0.001	0.001	0.001	0.001	1.9	3.2	2.2	2.2
split peas1	81.1	0.01	0.01	0.01	0.01	0.001	0.001	0.001	0.001	2.5	4.2	3.0	3.0
lima beans1	14.8	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	0.5	0.8	0.5	0.5
pinto beans1	36.1	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	1.1	1.9	1.3	1.3
soyabean sprouts1	12.10	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	0.4	0.6	0.4	0.4
					total	0.033	0.019	0.027	0.027	100	100.0	100.0	100.0

Estimated intake of coumestrol-lower

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Alfalfa sprouts1	46.8	0.47	0.19	0.36	0.36	0.022	0.009	0.017	0.017	68.1	46.3	62.1	62.1
Alfalfa tablets2	48.9					0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
Alfalfa and kelp2	847	0.01	0.01	0.01	0.01	0.008	0.008	0.008	0.008	26.2	44.1	31.2	31.2

kala chana seeds1,	61.3	0.01	0.01	0.01	0.01	0.001	0.001	0.001	0.001	1.9	3.2	2.3	2.3
split peas1	81.1	0.01	0.01	0.01	0.01	0.001	0.001	0.001	0.001	2.5	4.2	3.0	3.0
lima beans1	0	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
pinto beans1	36.1	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	1.1	1.9	1.3	1.3
soyabean sprouts1	4.50	0.01	0.01	0.01	0.01	0.000	0.000	0.000	0.000	0.1	0.2	0.2	0.2
					total	0.032	0.019	0.027	0.027	100	100.0	100.0	100.0

Estimated intake of diadzein-upper

	Conc total	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Ham	1.7	3.54	5.98	3.06	0	0.006	0.010	0.005	0.000	0.41	0.64	0.53	0.00
Saveloys	2.1	7.03	2.24	0.82	0	0.015	0.005	0.002	0.000	1.00	0.29	0.18	0.00
Sausages	4.2	28.04	19.23	9.5	0	0.118	0.081	0.040	0.000	7.99	5.06	4.09	0.00
Luncheon	3.2	1.16	2.87	1.21	0	0.004	0.009	0.004	0.000	0.25	0.58	0.40	0.00
Pies, savoury	6.8	38.58	38.58	16	0	0.092	0.092	0.038	0.000	6.23	5.75	3.90	0.00
Milk, soy	84.8	0.88	2.54	2.13	52.8	0.075	0.215	0.181	4.477	5.07	13.50	18.50	86.44
Milk, cow	0.33	365	235	173	160	0.120	0.078	0.057	0.053	8.18	4.86	5.85	1.02
Bread (soy component)	4.5	189	197	140	140	0.847	0.883	0.628	0.628	57.52	55.35	64.30	12.12
Beer	0.63	312	354	35	35	0.197	0.223	0.022	0.022	13.34	13.97	2.26	0.43
Total						1.47	1.60	0.98	5.18	100	100	100	100

Estimated intake of diadzein-lower

	Conc total	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Ham	1.7	3.54	5.98	3.06	0	0.006	0.010	0.005	0.000	0.6	0.9	0.7	0.0
Saveloys	0.5	7.03	2.24	0.82	0	0.004	0.001	0.000	0.000	0.4	0.1	0.1	0.0
Sausages	0.8	28.04	19.23	9.5	0	0.022	0.015	0.008	0.000	2.3	1.4	1.0	0.0
Luncheon	2.4	1.16	2.87	1.21	0	0.003	0.007	0.003	0.000	0.3	0.6	0.4	0.0
Pies, savoury	0.8	38.58	38.58	16	0	0.031	0.031	0.013	0.000	3.2	2.9	1.7	0.0
Milk, soy	62.2	0.88	2.54	2.13	52.8	0.055	0.158	0.132	3.284	5.6	14.6	17.2	84.4
Milk, cow	0.25	365	235	173	160	0.091	0.059	0.043	0.040	9.3	5.4	5.6	1.0

Bread (soy component)	4	189	197	140	140	0.756	0.788	0.560	0.560	77.8	73.4	73.3	14.5
Beer	0.02	312	354	35	35	0.006	0.007	0.001	0.001	0.6	0.7	0.1	0.0
Total						0.97	1.08	0.77	3.88	100	100	100	100

Estimated intake of enterodiol

	Conc total (mg/kg)	Consumption				Intake (mg/day)				%intake			
		YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Apples	0.01	50	39.3	32.1	25.7	0.0005	0.0004	0.0003	0.0003	0.3	0.3	0.3	0.1
Bananas	0.14	35.7	25	21.4	25.7	0.0050	0.0035	0.0030	0.0036	3.1	2.4	3.0	1.6
Bean, kidney	0.812	15	12.9	10.7	19.3	0.0122	0.0105	0.0087	0.0157	7.5	7.1	8.6	6.9
Beets	0.26	3.2	2.9	3.9	3.9	0.0008	0.0008	0.0010	0.0010	0.5	0.5	1.0	0.4
Bread or rolls,dark	0.23	51.4	52.5	30	125.7	0.0118	0.0121	0.0069	0.0289	7.3	8.2	6.9	12.7
Broccoli	0.65	4.4	5.1	4.6	10.2	0.0029	0.0033	0.0030	0.0066	1.8	2.2	3.0	2.9
Carrots	0.62	20.9	35.4	18.6	30.2	0.0130	0.0219	0.0115	0.0187	8.0	14.9	11.5	8.3
Cauliflower, brussel sprouts	0.77	4.4	5.1	4.6	10.2	0.0034	0.0039	0.0035	0.0079	2.1	2.7	3.5	3.5
Celery	0.14	2.9	2.9	5	2.9	0.0004	0.0004	0.0007	0.0004	0.3	0.3	0.7	0.2
Rolled oats	0.125	3.6	3.6	3.6	12.1	0.0005	0.0005	0.0005	0.0015	0.3	0.3	0.4	0.7
Coleslaw	0.282	17.9	22.1	27.1	15.2	0.0050	0.0062	0.0076	0.0043	3.1	4.2	7.6	1.9
Cucumber	0.11	6.4	5.7	6.4	8.6	0.0007	0.0006	0.0007	0.0009	0.4	0.4	0.7	0.4
Green beans	0.56	11.4	16.1	11.4	5.7	0.0064	0.0090	0.0064	0.0032	3.9	6.1	6.3	1.4
Green pepper	0.33	2.9	2.9	2.9	2.9	0.0010	0.0010	0.0010	0.0010	0.6	0.6	1.0	0.4
Mushrooms	0.13	1.8	1.8	1.8	5.3	0.0002	0.0002	0.0002	0.0007	0.1	0.2	0.2	0.3
Onions	1.01	2.9	2.9	2.9	6.4	0.0029	0.0029	0.0029	0.0065	1.8	2.0	2.9	2.8
Oranges	0.12	15.7	15	10.7	16.4	0.0019	0.0018	0.0013	0.0020	1.2	1.2	1.3	0.9
Peanuts, peanut butter	0.56	5.8	4.6	4.6	80.5	0.0032	0.0026	0.0026	0.0451	2.0	1.7	2.6	19.9
Pears	0.69	7.1	5.7	4.3	7.1	0.0049	0.0039	0.0030	0.0049	3.0	2.7	2.9	2.2
Pizza	0.035	17.9	16.1	14.3	0	0.0006	0.0006	0.0005	0.0000	0.4	0.4	0.5	0.0
Potatoes, fried	0.5	25.7	17.9	5.7	0	0.0129	0.0090	0.0029	0.0000	7.9	6.1	2.8	0.0
Potato, other	0.5	126.4	88.6	48.6	111.1	0.0632	0.0443	0.0243	0.0556	39.1	30.0	24.1	24.5

Potato, sweet	0.55	4.5	4.5	4.6	21.6	0.0025	0.0025	0.0025	0.0119	1.5	1.7	2.5	5.2
Salad, lettuce	0.55	3.6	3.4	3.2	2.9	0.0020	0.0019	0.0018	0.0016	1.2	1.3	1.7	0.7
Spaghetti and pasta with tomato sauce	0.036	7.1	5.7	4.3	4.3	0.0003	0.0002	0.0002	0.0002	0.2	0.1	0.2	0.1
Tomatoes	0.1	37.1	37.5	37.5	45.7	0.0037	0.0038	0.0038	0.0046	2.3	2.5	3.7	2.0
Total						0.1618	0.1477	0.1007	0.2268	100	100	100	100.0

Estimated intake of enterolactone

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Apples	0.34	50	39.3	32.1	25.7	0.017	0.013	0.011	0.0087	6.4	6.4	4.8	5.7
Bananas	0.55	35.7	25	21.4	25.7	0.020	0.014	0.012	0.0141	7.4	7.4	4.9	6.2
Bean, kidney	1.155	15	12.9	10.7	19.3	0.017	0.015	0.012	0.0223	6.6	6.6	5.3	6.5
Beets	1.09	3.2	2.9	3.9	3.9	0.003	0.003	0.004	0.0043	1.3	1.3	1.1	2.2
Bread or rolls,dark	0.648	51.4	52.5	30	125.7	0.033	0.034	0.019	0.0815	12.6	12.6	12.2	10.2
Broccoli	1.61	4.4	5.1	4.6	10.2	0.007	0.008	0.007	0.0164	2.7	2.7	2.9	3.9
Carrots	2.84	20.9	35.4	18.6	30.2	0.059	0.101	0.053	0.0858	22.5	22.5	36.0	27.8
Cauliflower, brussel sprouts	0.68	4.4	5.1	4.6	10.2	0.003	0.003	0.003	0.0069	1.1	1.1	1.2	1.6
Celery	0.17	2.9	2.9	5	2.9	0.000	0.000	0.001	0.0005	0.2	0.2	0.2	0.4
Rolled oats	0.351	3.6	3.6	3.6	12.1	0.001	0.001	0.001	0.0042	0.5	0.5	0.5	0.7
Coleslaw	0.249	17.9	22.1	27.1	15.2	0.004	0.006	0.007	0.0038	1.7	1.7	2.0	3.5
Cucumber	0.18	6.4	5.7	6.4	8.6	0.001	0.001	0.001	0.0015	0.4	0.4	0.4	0.6
Green beans	0.4	11.4	16.1	11.4	5.7	0.005	0.006	0.005	0.0023	1.7	1.7	2.3	2.4
Green pepper	1.62	2.9	2.9	2.9	2.9	0.005	0.005	0.005	0.0047	1.8	1.8	1.7	2.5
Mushrooms	0.43	1.8	1.8	1.8	5.3	0.001	0.001	0.001	0.0023	0.3	0.3	0.3	0.4
Onions	0.11	2.9	2.9	2.9	6.4	0.000	0.000	0.000	0.0007	0.1	0.1	0.1	0.2
Oranges	0.27	15.7	15	10.7	16.4	0.004	0.004	0.003	0.0044	1.6	1.6	1.4	1.5
Peanuts, peanut butter	1.05	5.8	4.6	4.6	80.5	0.006	0.005	0.005	0.0845	2.3	2.3	1.7	2.5
Pears	1.12	7.1	5.7	4.3	7.1	0.008	0.006	0.005	0.0080	3.0	3.0	2.3	2.5
Pizza	0.038	17.9	16.1	14.3	0	0.001	0.001	0.001	0.0000	0.3	0.3	0.2	0.3

Potatoes, fried	0.33	25.7	17.9	5.7	0	0.008	0.006	0.002	0.0000	3.2	3.2	2.1	1.0
Potato, other	0.33	126.4	88.6	48.6	111.1	0.042	0.029	0.016	0.0367	15.8	15.8	10.5	8.4
Potato, sweet	2.4	4.5	4.5	4.6	21.6	0.011	0.011	0.011	0.0518	4.1	4.1	3.9	5.8
Salad, lettuce	0.425	3.6	3.4	3.2	2.9	0.002	0.001	0.001	0.0012	0.6	0.6	0.5	0.7
Spaghetti and pasta with tomato sauce	0.039	7.1	5.7	4.3	4.3	0.000	0.000	0.000	0.0002	0.1	0.1	0.1	0.1
Tomatoes	0.11	37.1	37.5	37.5	45.7	0.004	0.004	0.004	0.0050	1.5	1.5	1.5	2.2
Total						0.264	0.280	0.190	0.452	100.0	100.0	100.0	100.0

Estimated intake of genistein-upper concentrations

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
Ham	4.2	3.54	5.98	3.06	0	0.015	0.025	0.013	0.000	0.6	0.9	0.9	0.0
Saveloys	5.4	7.03	2.24	0.82	0	0.038	0.012	0.004	0.000	1.5	0.5	0.3	0.0
Sausages	10.8	28.04	19.23	9.5	0	0.303	0.208	0.103	0.000	12.4	7.8	7.3	0.0
Luncheon	8.1	1.16	2.87	1.21	0	0.009	0.023	0.010	0.000	0.4	0.9	0.7	0.0
Pies, savoury	17.5	38.58	38.58	16	0	0.236	0.236	0.098	0.000	9.6	8.9	7.0	0.0
Milk, soy	110	0.88	2.54	2.13	52.8	0.097	0.279	0.234	5.808	3.9	10.5	16.6	86.0
Bread (soy component)	6.3	189	197	140	140	1.191	1.242	0.882	0.882	48.6	46.6	62.7	13.1
Beer	1.8	312	354	35	35	0.562	0.637	0.063	0.063	22.9	23.9	4.5	0.9
Total						2.45	2.66	1.41	6.75		100.0	100.0	100.0

Estimated intake of genistein-lower concentrations

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF

Ham	4.2	3.54	5.98	3.06	0	0.015	0.025	0.013	0.000	1.1	1.7	1.3	0.0
Saveloys	1.3	7.03	2.24	0.82	0	0.009	0.003	0.001	0.000	0.7	0.2	0.1	0.0
Sausages	2.2	28.04	19.23	9.5	0	0.061	0.042	0.021	0.000	4.4	2.8	2.0	0.0
Luncheon	6.1	1.16	2.87	1.21	0	0.007	0.017	0.007	0.000	0.5	1.2	0.7	0.0
Pies, savoury	1.9	38.58	38.58	16	0	0.075	0.075	0.031	0.000	5.4	5.0	3.0	0.0
Milk, soy	39.0	0.88	2.54	2.13	52.8	0.034	0.099	0.083	2.059	2.5	6.6	8.1	70.4
Bread (soy component)	6.2	189	197	140	140	1.168	1.217	0.865	0.865	84.4	81.5	84.6	29.6
Beer	0.0	312	354	35	35	0.014	0.016	0.002	0.002	1.0	1.1	0.2	0.1
Total						1.38	1.49	1.02	2.93	100.0	100.0	100.0	100.0

Estimated intake of isoliquiritigenin

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
Licorice	9600	0.24	0.24	0.24	0.24	2.304	2.304	2.304	2.304	100.0	100.0	100.0	100.0

Estimated intake of keampferol

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
broccoli	72	8.8	10.2	19.5	10.2	0.634	0.734	1.404	0.734	35.0	21.0	28.0	24.6
endive	46	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
leek	30	1	1	0.67	0.67	0.030	0.030	0.020	0.020	1.7	0.9	0.4	0.7
strawberry	12	1.47	1.47	1.64	1.64	0.018	0.018	0.020	0.020	1.0	0.5	0.4	0.7
Tea1	14	80.7	194.3	255	158	1.130	2.720	3.570	2.212	62.4	77.7	71.2	74.1
						1.81	3.50	5.01	2.99	100.0	100.0	100.0	100.0

Estimated intake of luteolin

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
celery	22	2.9	2.9	5	2.9	0.064	0.064	0.110	0.064	66.7	66.7	77.5	66.7

Red pepper	11	2.9	2.9	2.9	2.9	0.032	0.032	0.032	0.032	33.3	33.3	22.5	33.3
						0.096	0.096	0.142	0.096	100.0	100.0	100.0	100.0

Estimated intake of naringenin

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
Grapefruit	480	2.9	2.9	1.8	1.8	1.392	1.392	0.864	0.864	48.8	48.8	47.4	47.4
Grapefruit juice	384	3.8	3.8	2.5	2.5	1.459	1.459	0.960	0.960	51.2	51.2	52.6	52.6
						2.85	2.85	1.82	1.82	100.0	100.0	100.0	100.0

Estimated intake of nonylphenols

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
mayonnaise	5	0.44	0.44	0.44	0.44	0.002	0.002	0.002	0.002	0.0	0.1	0.1	0.1
sugar	6.8	13.7	13.7	13.7	13.7	0.093	0.093	0.093	0.093	2.0	2.6	3.1	2.7
tuna	8.1	0.7	0.7	0.7	0	0.006	0.006	0.006	0.000	0.1	0.2	0.2	0.0
butter	14.4	18.9	15.9	10.3	12	0.272	0.229	0.148	0.173	5.8	6.4	4.9	5.0
lard	10.2	5.2	5.2	5.2	5.2	0.053	0.053	0.053	0.053	1.1	1.5	1.8	1.5
spinach	1.3	1.3	1.3	1.3	1.3	0.002	0.002	0.002	0.002	0.0	0.0	0.1	0.0
milk chocolate	14.1	2.9	2.9	1.8	2.8	0.041	0.041	0.025	0.039	0.9	1.1	0.8	1.1
egg	1.5	16.5	21.8	13.8	22.7	0.025	0.033	0.021	0.034	0.5	0.9	0.7	1.0
pineapple	2.6	2.1	3.6	3.6	4.3	0.005	0.009	0.009	0.011	0.1	0.3	0.3	0.3
fresh cheese	7.5	29.9	20	18.9	32.9	0.224	0.150	0.142	0.247	4.8	4.2	4.7	7.1
pasta	1	11.4	11.4	8.6	27.9	0.011	0.011	0.009	0.028	0.2	0.3	0.3	0.8
apples	19.4	114.3	67.1	57.1	75	2.217	1.302	1.108	1.455	47.2	36.3	36.8	41.7
chicken meat	3.8	35.4	35.4	27.5	0	0.135	0.135	0.105	0.000	2.9	3.7	3.5	0.0
tea	0.1	80.7	194	255	158	0.008	0.019	0.026	0.016	0.2	0.5	0.8	0.5
potatoes	0.6	130	93.2	51.1	111.1	0.078	0.056	0.031	0.067	1.7	1.6	1.0	1.9
tomatoes	18.5	37.1	37.5	37.5	40	0.686	0.694	0.694	0.740	14.6	19.3	23.0	21.2
whole milk	1.1	399	256	190	200	0.439	0.282	0.209	0.220	9.4	7.8	6.9	6.3
wholemeal bread	1.6	51.4	52.5	30	125.7	0.082	0.084	0.048	0.201	1.8	2.3	1.6	5.8

beer	0.5	186	271	19.3	28.6	0.093	0.136	0.010	0.014	2.0	3.8	0.3	0.4
coffee(brewed)	0.3	128	241	278	220	0.038	0.072	0.083	0.066	0.8	2.0	2.8	1.9
orange juice	0.1	50	35	25.7	21.4	0.005	0.004	0.003	0.002	0.1	0.1	0.1	0.1
freshwater fish	2020	0.08	0.08	0.08	0	0.162	0.162	0.162	0.000	3.4	4.5	5.4	0.0
water	0.029	547	541	877	886	0.016	0.016	0.025	0.026	0.3	0.4	0.8	0.7
						4.69	**3.59**	**3.01**	**3.49**	100	100	100	100

Estimated intake of PCBs

	Conc (ng/kg)	Consumption (g/day)				Intake (mg/day)				%intake			
		YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
Beef Meat	58.7	26.4	25.7	15.7	0	1.55	1.51	0.92	0	1.7	1.8	1.6	0.0
Sheep meat	67.6	40	64.3	23.6	0	2.71	4.35	1.60	0	3.0	5.2	2.8	0.0
Pork meat	535.9	18.6	16.4	12.1	0	9.97	8.79	6.48	0	11.1	10.5	11.2	0.0
Beef fat	638.6	3.5	3.4	2.1	0	2.23	2.17	1.32	0	2.5	2.6	2.3	0.0
Sheep fat	198.3	6.2	10.0	3.7	0	1.23	1.98	0.73	0	1.4	2.4	1.3	0.0
Pork fat	194.9	4.6	4.1	3.0	0	0.90	0.79	0.58	0	1.0	0.9	1.0	0.0
Liver	1041	2.5	2.1	1.8	0	0.26	0.22	0.19	0	0.3	0.3	0.3	0.0
Processed meats	117.5	23.6	22.9	13.6	0	2.77	2.69	1.60	0	3.1	3.2	2.8	0.0
Milk	14.9	365	235.4	172.9	160	5.43	3.51	2.57	2.38	6.1	4.2	4.5	5.4
Butter	514.1	18.9	15.9	10.3	12	9.72	8.17	5.30	6.17	10.8	9.8	9.2	14.0
Cheese	237.5	29.9	20	18.9	32.9	7.10	4.75	4.49	7.81	7.9	5.7	7.8	17.7
Ice cream/yoghurt	83.6	33.9	20.9	17.1	40.3	2.83	1.75	1.43	3.37	3.2	2.1	2.5	7.6
New Zealand fish	552.8	11.2	16.3	6.9	0	6.19	9.01	3.81	0.00	6.9	10.8	6.6	0.0
Imported tinned fish	2371.4	5.1	5.4	5	0	12.09	12.81	11.86	0.00	13.5	15.3	20.5	0.0
Shellfish	231.7	5	5	5	0	1.16	1.16	1.16	0.00	1.3	1.4	2.0	0.0
Poultry	27.0	20	20	14.3	0	0.54	0.54	0.39	0.00	0.6	0.6	0.7	0.0
Eggs	141.9	16.5	21.8	13.9	22.7	2.34	3.09	1.97	3.22	2.6	3.7	3.4	7.3
Bread	43.2	189.8	152.8	105.4	205.7	8.20	6.60	4.55	8.88	9.1	7.9	7.9	20.1
Cereals	70.1	24.5	18.9	16.4	48.2	1.72	1.32	1.15	3.38	1.9	1.6	2.0	7.7
Potatoes	52.1	155.7	111.1	56.8	111.1	8.11	5.79	2.96	5.79	9.0	6.9	5.1	13.1
Snack foods	161.9	14.6	14.6	14.6	14.6	2.36	2.36	2.36	2.36	2.6	2.8	4.1	5.4
Vegetable fats/oils	37.9	10.3	10.3	10.3	19.2	0.39	0.39	0.39	0.73	0.4	0.5	0.7	1.6

Daily intake (ng/day)	0	89.80	83.74	57.81	44.09	100	100	100	100

Estimated intake of phloretin

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF	YM	24+M	24+ F	VF
apples	40	50	39.3	32.1	25.7	2.000	1.572	1.284	1.028	29.3	42.9	40.6	21.8
Apple juice1	75	64.3	27.9	25	49.3	4.823	2.093	1.875	3.698	70.7	57.1	59.4	78.2
						6.82	3.66	3.16	4.7	100.0	100.0	100.0	100.0

Estimated intake of quercetin-upper

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
apples	36	50	39.3	32.1	25.7	1.800	1.415	1.156	0.925	25.5	10.1	8.5	9.7
Apple juice1	2.8	64.3	27.9	25	49.3	0.180	0.078	0.070	0.138	2.5	0.6	0.5	1.4
apricot	25	30	26.1	26.4	69.2	0.750	0.653	0.660	1.730	10.6	4.7	4.9	18.1
Broad beans	20	0.35	0.35	0.35	0.35	0.007	0.007	0.007	0.007	0.1	0.1	0.1	0.1
broccoli	30	4.4	5.1	4.6	10.2	0.132	0.153	0.138	0.306	1.9	1.1	1.0	3.2
grape	15.0	2.4	2.4	2.4	2.4	0.036	0.036	0.036	0.036	0.5	0.3	0.3	0.4
Green beans	39	11.4	16.1	11.4	5.7	0.445	0.628	0.445	0.222	6.3	4.5	3.3	2.3
Green pepper	18	2.9	2.9	2.9	2.9	0.052	0.052	0.052	0.052	0.7	0.4	0.4	0.5
lettuce	14	3.6	3.4	3.2	2.9	0.050	0.048	0.045	0.041	0.7	0.3	0.3	0.4
onion	347	2.9	2.9	2.9	6.4	1.006	1.006	1.006	2.221	14.2	7.2	7.4	23.3
Orange juice	5.7	27.1	19.3	15	21.4	0.154	0.110	0.086	0.122	2.2	0.8	0.6	1.3
pear	6.4	7.1	5.7	4.3	7.1	0.045	0.036	0.028	0.045	0.6	0.3	0.2	0.5
plum	9	2.3	2.3	2.3	2.3	0.021	0.021	0.021	0.021	0.3	0.1	0.2	0.2
Red wine	11	8.6	19.3	12.1	11.4	0.095	0.212	0.133	0.125	1.3	1.5	1.0	1.3
strawberry	8.6	1.47	1.47	1.64	1.64	0.013	0.013	0.014	0.014	0.2	0.1	0.1	0.1
Tea1	20	103	463.5	472.8	158	2.060	9.270	9.456	3.150	29.1	66.3	69.6	33.0

tomato	8	27.1	28.2	28.9	27.1	0.217	0.226	0.231	0.217	3.1	1.6	1.7	2.3
Tomato juice1	13	0.7	0.7	0.7	12.9	0.009	0.009	0.009	0.168	0.1	0.1	0.1	1.8
						6.86	8.85	9.77	9.54	100.0	100.0	100.0	100.0

Estimated intake of quercetin-lower

	Conc	Consumption (g/day)				Intake (mg/day)				%intake			
	(mg/kg)	YM	24+M	24+ F	VF	YM	24+ M	24+ F	VF	YM	24+ M	24+ F	VF
apples	36	50	39.3	32.1	25.7	1.800	1.415	1.156	0.925	27.0	16.4	12.0	9.8
Apple juice1	2.8	64.3	27.9	25	49.3	0.180	0.078	0.070	0.138	2.7	0.9	0.7	1.5
apricot	25	30	26.1	26.4	69.2	0.750	0.653	0.660	1.730	11.2	7.6	6.9	18.4
Broad beans	20	0.35	0.35	0.35	0.35	0.007	0.007	0.007	0.007	0.1	0.1	0.1	0.1
broccoli	30	4.4	5.1	4.6	10.2	0.264	0.306	0.585	0.306	4.0	3.5	6.1	3.2
grape	12.0	2.4	2.4	2.4	2.4	0.029	0.029	0.029	0.029	0.4	0.3	0.3	0.3
Green beans	29	11.4	16.1	11.4	5.7	0.331	0.467	0.331	0.165	5.0	5.4	3.4	1.8
Green pepper	18	2.9	2.9	2.9	2.9	0.052	0.052	0.052	0.052	0.8	0.6	0.5	0.6
lettuce	14	3.6	3.4	3.2	2.9	0.050	0.048	0.045	0.041	0.8	0.6	0.5	0.4
onion	347	2.9	2.9	2.9	6.4	1.006	1.006	1.006	2.221	15.1	11.7	10.5	23.6
Orange juice	3.4	27.1	19.3	15	21.4	0.092	0.066	0.051	0.073	1.4	0.8	0.5	0.8
pear	6.4	7.1	5.7	4.3	7.1	0.045	0.036	0.028	0.045	0.7	0.4	0.3	0.5
plum	9	2.3	2.3	2.3	2.3	0.021	0.021	0.021	0.021	0.3	0.2	0.2	0.2
Red wine	11	8.6	19.3	12.1	11.4	0.095	0.212	0.133	0.125	1.4	2.5	1.4	1.3
strawberry	8.6	1.47	1.47	1.64	1.64	0.013	0.013	0.014	0.014	0.2	0.1	0.1	0.1
Tea1	20	103	463.5	472.8	158	1.614	3.886	5.100	3.150	24.2	45.0	53.1	33.4
tomato	8	27.1	28.2	28.9	27.1	0.142	0.157	0.186	0.217	2.1	1.8	1.9	2.3
Tomato juice1	13	0.7	0.7	0.7	12.9	0.186	0.186	0.139	0.168	2.8	2.2	1.4	1.8
						6.68	8.64	9.61	9.43	100.0	100.0	100.0	100.0

Appendix 2: Simulated typical diets for the 1997/1998 Total Diet Survey

Table A1: Summary of weights of foods consumed (Brinsdon S, Nicolson R, Mackay S, 1999)

ID	Food	19-24 male	25+ male	25+ female	19-40 female lacto-ovo vegetarian	4-6 child	1-3 child
		Total grams (or millilitres) for the 14 day diet					
A	**Grains**						
1	Bran cereal	20	20	20	85	10	10
2	Cornflakes	61	40	60	60	50	50
3	Muesli	30	25	25	90	25	25
4	Rolled Oats	50	50	50	170	30	25
5	Wheatbix	182	150	75	270	100	75
6	Cake plain	481	380	355	200	210	150
7	Chocolate biscuit	280	250	175	108	240	195
8	Cracker biscuit	296	270	135	230	85	55
9	Sweet biscuit	140	155	85	90	210	145
10	Flour, white	75	80	75	145	45	40
11	Noodles, instant	30	30	60	100	50	40
12	Rice, white	60	100	60	270	50	40
13	Spaghetti, dried	80	80	60	330	50	40
14	Mixed grain bread	400	350	240	870	280	160
15	Wheatmeal bread	320	385	180	890	180	110
16	White bread	1937	1405	1055	1120	1160	665
17	Spaghetti in sauce	100	80	60	60	70	35
B	**Dairy Products**						
18	Butter, salted	265	223	144	168	129	86
19	Cheese	418	280	265	460	185	145
20	Dairy dessert	160	110	60	150	150	80
21	Ice cream	270	160	140	170	155	135
22	Milk, trim	520	685	790	1150	600	350
23	Milk, standard	4590	2610	1630	1090	6100	5465
24	Yoghurt	205	133	100	395	350	190
C	**Oils**						
25	Olive oil	22	9	19	80	9	8
26	Margarine	174	146	87	124	67	44
27	Salad/Cooking Oil	27	18	25	65	11	6
D	**Chicken, Eggs, Fish and Meat**						
28	Bacon	60	70	50	0	35	20
29	Beef mince	190	180	110	0	50	40
30	Beef rump steak	180	180	110	0	55	20
31	Chicken	170	180	140	0	55	35
32	Egg	231	305	194	318	185	170
33	Fish, terakihi	70	120	60	0	30	20
34	Lamb, mutton leg	190	180	130	0	55	25
35	Lamb mutton shoulder	370	360	200	0	80	50

ID	Food	19-24 male	25+ male	25+ female	19-40 female lacto-ovo vegetarian	4-6 child	1-3 child
36	Luncheon	150	140	105	0	55	35
37	Pork pieces	200	160	120	0	37	35
38	Sausages, beef	180	180	85	0	50	30
39	Salmon, canned	72	75	70	0	25	25
40	Soup, chicken	200	200	180	0	80	100
E	**Vegetables**						
41	Beans	160	225	160	80	95	90
42	Broccoli, cauliflower	123	143	130	143	60	45
43	Cabbage	250	310	380	213	115	70
44	Capsicum	40	40	40	40	15	10
45	Carrots	293	495	260	423	155	120
46	Celery	40	40	70	40	30	25
47	Courgette	30	30	30	60	20	15
48	Cucumber	90	80	90	120	20	20
49	Kumara	63	63	65	303	40	40
50	Lettuce	50	47	45	40	12	10
51	Mushrooms	25	25	25	75	10	10
52	Onions	40	40	40	90	15	12
53	Peas	258	395	325	348	140	120
54	Potato, boiled	1240	860	440	560	415	265
55	Potato, baked	530	380	240	896	230	130
56	Potato crisps	50	65	35	100	45	35
57	Pumpkin	70	80	65	110	105	135
58	Silverbeet	160	145	155	30	35	35
59	Tomato	180	195	255	320	65	65
60	Baked beans	210	180	150	270	35	35
61	Beetroot	45	40	55	45	25	25
62	Corn	73	73	80	200	45	40
63	Pasta sauce	70	50	50	80	60	40
64	Tomato sauce	70	80	70	60	80	70
65	Tomatoes in juice	200	200	150	180	80	80
F	**Fruits**						
66	Juice, apple based	900	390	350	690	260	130
67	Apple	700	550	450	360	250	170
68	Bananas	500	350	300	360	200	200
69	Kiwifruit	100	100	100	200	70	60
70	Orange	220	210	150	230	140	130
71	Orange juice	380	270	210	300	200	160
72	Pears	100	80	60	100	90	60
73	Nectarines	130	110	110	230	60	60
74	Apricots, stewed	420	365	370	600	240	200
75	Dates	20	20	15	70	15	10
76	Peaches, canned	230	190	225	240	130	120
77	Pineapple, canned	30	50	50	60	30	30
78	Raisins/Sultanas	25	15	20	50	20	10
G	**Spreads and Sweets**						

ID	Food	19-24 male	25+ male	25+ female	19-40 female lacto-ovo vegetarian	4-6 child	1-3 child
79	Mints, confectionery	30	20	20	20	20	20
80	Chocolate	40	40	25	40	25	25
81	Honey	10	15	10	15	7	7
82	Jam	45	48	65	58	15	10
83	Marmalade	25	17	25	20	5	5
84	Yeast extract	17	17	22	17	15	15
85	Jelly	300	300	300	150	220	180
H	**Alcohol**						
86	Beer, draught	1600	1900	120	200	0	0
87	Beer, lager	1002	1900	150	200	0	0
88	Wine, red	120	270	170	160	0	0
89	Wine, white	150	220	300	330	0	0
I	**Take-Aways**						
90	Chicken nuggets	110	100	60	0	45	30
91	Chinese dish	200	150	120	0	80	40
92	Fish in batter	145	180	60	0	40	25
93	Hamburger	270	225	225	0	150	60
94	Meat pie	160	160	120	0	120	40
95	Pizza	250	225	200	0	100	80
96	Hot potato chips	360	250	80	0	50	30
J	**Nuts**						
97	Peanut butter	45	35	40	85	17	10
98	Peanuts	36	30	24	76	7	0
K	**Beverages**						
99	Lemonade	484	360	120	234	400	240
100	Coke	925	655	225	225	100	100
101	Milo - made up	440	440	440	660	520	450
102	Coffee brewed	1790	3370	3900	3080	0	0
103	Breakfast drink	420	300	350	150	1200	920
104	Tea infused	1130	2720	3570	2205	0	0
105	Drinking water*	7653	7580	12281	12410	9073	6035
L	**Additional meat and shellfish**						
106	Lamb's liver	35	30	25	0	20	10
107	Mussels	35	35	35	0	0	0
108	Oysters	35	35	35	0	0	0
M	**Vegetarian additions**						
109	Hummus	0	0	0	295	0	0
110	Soya milk	0	0	0	740	0	0
111	Tofu	0	0	0	265	0	0
O	**Children's Foods/Snacks**						
112	Cheese flav snacks	0	0	0	0	90	55
113	Fish fingers	0	0	0	0	40	20
114	Tortilla chips	0	0	0	0	45	30

* This is additional drinking water and does not include that used in making up drinks.

Appendix 3: An example Excel spreadsheet used for processing yeast assay data

Run #20 Estradiol std @ 54.48µg/L													
well conc (ng/L)		2724	1362	681	340	170	85.1	42.6	21.3	10.6	5.32	2.66	1.33	
well conc (nM)		10	5	2.5	1.25	0.625	0.3125	0.156	0.0781	0.0391	0.0195	0.0098	0.0049	
48 hrs	Well# 1		2	3	4	5	6	7	8	9	10	11	**12**	
Plate #1														
540nm row B		2.941	3	2.87	2.653	2.326	1.845	1.426	1.185	1.147	1.133	1.094	1.056	
610nm		1.407	1.436	1.425	1.305	1.235	1.147	1.054	0.979	1.007	1.016	0.993	0.96	
abs 540-(abs 610-abs 610 bl)		2.512333	2.542333	2.423333	2.326333	2.06933	1.67633	1.35033	1.18433	1.11833	1.09533	1.07933	1.07433	
blank610nm		1.03	1.027	0.98	0.975	0.946	1.008	0.964	0.983	0.924	0.98	0.944	0.979	0.97833
540nm row E		3	2.849	2.87	2.627	2.333	1.936	1.535	1.298	1.168	1.132	1.003	1.034	
610nm		1.426	1.386	1.379	1.333	1.241	1.191	1.081	1	1.012	1.014	0.914	0.947	
abs 540-(abs 610-abs 610 bl)		2.552333	2.441333	2.469333	2.272333	2.07033	1.72333	1.43233	1.27633	1.13433	1.09633	1.06733	1.06533	
blank610nm		1.03	1.027	0.98	0.975	0.946	1.008	0.964	0.983	0.924	0.98	0.944	0.979	0.97833
Plate #2														
540nm row B		2.941	2.893	2.709	2.569	2.154	1.823	1.401	1.259	1.106	1.046	1.065	1.015	
610nm		1.392	1.324	1.329	1.261	1.192	1.076	0.999	0.954	0.935	0.918	0.97	0.938	
abs 540-(abs 610-abs 610 bl)		2.575833	2.595833	2.406833	2.334833	1.98883	1.77383	1.42883	1.33183	1.19783	1.15483	1.12183	1.10383	
blank610nm		1.062	1.041	1.045	1.012	1.055	1.028	0.958	1.016	1.015	1.036	1.026	1.028	1.02683
540nm row E		2.849	2.615	2.653	2.499	2.258	1.888	1.517	1.299	1.094	1.074	0.997	1.008	
610nm		1.317	1.251	1.251	1.246	1.184	1.116	1.026	0.987	0.915	0.945	0.904	0.918	
abs 540-(abs 610-abs 610 bl)		2.558833	2.390833	2.428833	2.279833	2.10083	1.79883	1.51783	1.33883	1.20583	1.15583	1.11983	1.11683	
blank610nm		1.062	1.041	1.045	1.012	1.055	1.028	0.958	1.016	1.015	1.036	1.026	1.028	1.02683

Cont'd

estradiol nmoles 48 hrs

	plate #1 B	plate #1 E	plate #2 B	plate #2 E	mean	SE	1/[S]	1/mean
0.0049	1.074333	1.065333	1.1038	1.116833	1.09008	0.02426	204.082	0.91736
0.0098	1.079333	1.067333	1.1218	1.119833	1.09708	0.02787	102.041	0.91151
0.0195	1.095333	1.096333	1.1548	1.155833	1.12558	0.03436	51.2821	0.88843
0.0391	1.118333	1.134333	1.1978	1.205833	1.16408	0.0442	25.5754	0.85905
0.0781	1.184333	1.276333	1.3318	1.338833	1.28283	0.07137	12.8041	0.77952
0.156	1.350333	1.432333	1.4288	1.517833	1.43233	0.06843	6.41026	0.69816
0.3125	1.676333	1.723333	1.7738	1.798833	1.74308	0.05446	3.2	0.5737
0.625	2.069333	2.070333	1.9888	2.100833	2.05733	0.04795	1.6	0.48607
1.25	2.326333	2.272333	2.3348	2.279833	2.30333	0.0318	0.8	0.43415
2.5	2.423333	2.469333	2.4068	2.428833	2.43208	0.02653	0.4	0.41117
5	2.542333	2.441333	2.5958	2.390833	2.49258	0.0933	0.2	0.40119
10	2.512333	2.552333	2.5758	2.558833	2.54983	0.02689	0.1	0.39218

Y50 1.81996

[Equation]

f=y0+a/(1+exp(-(x-x0)/b))

R = 0.99865381 Rsqr = 0.99730943 Adj Rsqr = 0.996

Coefficient	Std. Error	t	P
a	40.2287	324.849	0.123
b	0.5176	0.1148	4.507
x0	-1.7182	4.9505	-0.347
y0	-37.743	324.841	-0.116
EC50	0.396	1.81988	